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MULTIFACTORIAL MODULATION OF THE BLOOD-BRAIN BARRIER:
RELATIONSHIP TO STROKE

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Medicine, Graduate Center for Nutritional Sciences
at the University of Kentucky

By
Bei Zhang
Lexington, Kentucky

Director: Dr. Michal Toborek, Professor of Nutritional Sciences

Lexington, Kentucky

2013

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ABSTRACT OF DISSERTATION

MULTIFACTORIAL MODULATION OF THE BLOOD-BRAIN BARRIER: RELATIONSHIP TO STROKE

The blood-brain barrier (BBB) is a dynamic interface, mainly consisting of highly specialized brain microvascular endothelial cells (BMECs) that segregate the central nervous system (CNS) from the peripheral circulation. Impairment of the BBB, due to disruption of tight junction (TJ) proteins and inflammatory responses, may initiate and/or contribute to the progress of CNS disorders, including stroke. Stroke is the second leading cause of death worldwide. It has been shown that aging and environmental pollutants can induce brain endothelium dysfunction, and are considered as risk factors for stroke.

Deficiency of telomerase is highly linked with aging-associated vascular diseases. Evidence indicates that patients with shorter telomere length are at higher risk of heart disease or stroke. Results in this dissertation address the influence of telomerase reverse transcriptase (TERT), a key component of telomerase, on the BBB integrity in the context of ischemic stroke induced brain injury. Our results indicate that aging-related BBB alterations aggregate the stroke outcomes by inducing oxidative stress and stimulating proinflammatory responses on the brain microvessels.

The ability of the BBB to protect the brain from harmful compounds indicates that the BBB may be targeted by chemical toxicants in the peripheral circulation. Polychlorinated biphenyls (PCBs) are persistent organic pollutants that frequently bind to nanoparticles (NPs) in the environment. Our results demonstrate that binding PCB153, one of the most abundant PCB congeners in the environment, to silica nanoparticles (PCB153-NPs) potentiates cerebrovascular toxicity and stroke outcomes via stimulation of inflammatory responses and disruption of BBB integrity. These events are mediated by activation of toll-like receptor 4 (TLR4), which subsequently recruits tumor necrosis factor-associated factor 6 (TRAF6) and initiates the production of multiple inflammatory mediators.

Research presented in this dissertation demonstrates that aging and environmental pollutants play crucial roles in modifying the function of the BBB through alterations of inflammatory responses and TJ protein expression, which further contribute to the progression of stroke-induced cerebral ischemic injury.

KEYWORDS: Blood-brain barrier, Stroke, Telomere, Polychlorinated biphenyls, Toll-like receptor 4

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Chapter one: Introduction

1. Background

1.1. Pathology of Stroke

1.1.1. Overview

Stroke, which refers to a diseased condition caused by the occlusion or hemorrhage of blood cerebral vessels, is the second leading cause of death worldwide [1]. Over 5.7 million people die from stroke in every year, and more than 85% of these deaths are in developing countries [1]. According to data from 2006, stroke accounted for roughly 1 of each 18 deaths in the United States. About every 40 seconds, someone in the United States has a stroke [2]. The report of the National Institute of Neurological and Communicative Disorders and Stroke (NINCDS) data bank showed that 70% of strokes are caused by cerebral ischemia, 27% are due to cerebral hemorrhage, and 3% are caused by some unknown reasons [3]. Stroke can be classified into four major types: primary intracerebral hemorrhage, subarachnoid hemorrhage, ischemic stroke and undetermined stroke. The etiologies of stroke include atherothrombosis of large arteries, embolism from the heart or cerebropetale arteries, sudden occlusion of small penetrating arteries (lacunar stroke), arteritis, arterial dissection, and various genetic and hematological disorders [4]. Ischemic stroke can be triggered by in situ thrombosis, embolism, or relative hypoperfusion. In all cases, stroke induces brain cell dysfunction, neurological deficits, and ultimately leading to death.

1.1.2. Risk factors

Hypertension, hyperlipidemia, atrial fibrillation, and myocardial infarction are the primary risk factors for the atherothrombotic stroke, cardioembolic stroke and primary intracerebral hemorrhage. Several others, classified as modifiable and non-modifiable factors, also tend to increase the incidence of stroke. For example, risk factors like age, gender, ethnicity, and heredity have been identified as non-modifiable, whereas numerous modifiable factors, such as smoking, alcohol intake, daily diet and physical

activity, are considered as targets to address the prevention of stroke in the appropriate perspectives. Additionally, environmental pollution is also recognized as a risk factor for vascular disease and stroke [5].

Age is recognized as the most significant risk factor for stroke. After the age of 55, the rate of stroke rises by a factor larger than two for each consecutive 10 years in both sexes [6]. Age-related brain changes are associated with increased blood-brain barrier (BBB) permeability, which is proposed as an important mechanism in the initiation or aggravation of cerebral microvascular disease. Animal studies have shown that the initial step in the lacunar stroke is plasma leakage into the vessel wall and surrounding brain parenchyma [7]. With normal aging, the brain capillary surface area reduces while the capillary diameter, length, and volume increase. This degeneration of the brain vascular structure and function may lead to lipohyalinosis, lumen narrowing, and blood flow reduction. Cerebral hypoperfusion leads to oligemia and subsequent disruption of the microcirculation, which further induce the BBB damage and enhance the intrinsic susceptibility of brain cells to ischemic injury [8, 9].

Recent evidence indicates that environmental pollution is a risk factor for vascular disease and stroke. The most important environmental pollutants include ambient particulate matter [10] and persistent organic pollutants (POPs), such as chlorinated pesticides, dioxin and polychlorinated biphenyls (PCBs) [11, 12]. Because of their lipophilicity and resistance to degradation, chlorinated organic compounds have high tendency to accumulate in the human body, and thus increase the vascular risks of hypertension, diabetes mellitus, ischemic heart disease, and atherosclerosis. Laboratory evidence demonstrated that animals exposed to PCBs show elevation of triglycerides and serum lipids. Exposure to dioxin induces endothelium release of reactive species, which promote adhesion of foam cells and formation of atherosclerosis with the vascular wall lesions in brain, heart, and renal arteries [13]. Additionally, an epidemiological study indicated that living close to POP contaminated waste sites is significantly associated with increased risk of ischemic stroke [14].

1.1.3. Risk factor-induced alteration of cerebral blood vessels

Stroke risk factors alter the structure and function of blood vessels by promoting atherosclerosis and stiffening of arteries, as well as by inducing narrowing, thickening, and tortuosity of arterioles and capillaries. These morphological changes in cerebral vasculature are often associated with regulation and/or reduction in cerebral blood flow (CBF). Endothelial dysfunction (ED) is associated with aging and other risk factors and is associated with vascular-related diseases, such as stroke[15]. ED is characterized by increased permeability, altered vasodilatation, increased vascular reactivity, platelet activation, and enhanced thrombogenicity, as well as leukocyte adhesion and monocyte migration. Increased oxidative stress and inflammation in the vascular wall are the main causes for ED. Under oxidative stress, biological inactivation of nitric oxide (NO) by the superoxide weakens the beneficial effect of NO-mediated regulation of vascular tone, which subsequently leads to the production of the deleterious peroxynitrite and vasoconstriction [16, 17]. Loss of the protective effects of NO also promotes platelet aggregation, leukocyte adhesion, and smooth muscle proliferation, which are key steps in the formation of vascular inflammation [18]. Additionally, reactive oxygen species (ROS) can induce inflammation by increasing BBB permeability through upregulation of vascular endothelial growth factor (VEGF) and triggering activation of NF- κ B, which promotes the expression of cytokines, matrix remodeling enzymes, and proinflammatory genes. Matrix remodeling, together with smooth muscle cell migration and intimal hyperplasia which are also provoked by oxidative stress and inflammation, are critical factors for atherosclerosis and vascular structure alterations [19]. Thus, risk factors like aging and POPs may exert deleterious effects on cerebral blood vessels through oxidative stress and vascular inflammation; however, it remains to be determined how individual risk factor triggers the activation of one or both of these mechanisms.

1.1.4. Mechanisms of ischemic brain injury

Ischemic stroke begins with severe focal hypoperfusion. The ischemic core is the rapidly and irreversibly injured territory with severely impaired blood flow. The primary

mechanism of cell death in the ischemic core is energy failure. At the periphery region of ischemic core, where the blood flow arises from adjacent vascular territories, the so-called ischemic penumbra, cell death develops more slowly because of partially preserved energy metabolism. In the ischemic penumbra, several deleterious mechanisms contribute to the ongoing cellular injury and infarct progression, which ultimately result in the demise of the ischemic penumbra within hours after stroke onset [20].

1.1.4.1. Energy failure, excitotoxicity and ionic imbalance

Brains consume relatively high content of oxygen and glucose as compared to other organs. The energy supply for a functional brain is almost exclusively from oxidative phosphorylation to generate adenosine triphosphate (ATP) [21]. The first consequence of focal hypoperfusion is the depletion of essential substrates, especially oxygen and glucose, which subsequently causes the failure of ATP production for energy. In addition, depletion of oxygen supply to the brain leads to anaerobic glycolysis and lactate accumulation [22, 23]. Elevation of lactate level is considered as a possible cause of secondary damage which leads to increased infarct volume of the brain and neurological deficits [24, 25]. The failure of cellular bioenergetics quickly results in depolarization of energy-dependent ion channels on the neurons and glia [26, 27], followed by the activation of somatodendritic and presynaptic voltage-dependent Ca^{2+} channels and the release of excitatory amino acids into the extracellular space. The increase of extracellular excitatory neurotransmitters, especially glutamate, overstimulates the glutamate receptors, such as amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), kainate and N-methyl-d-aspartic acid (NMDA), which consequently leads to influx and build-up of intracellular Na^+ , Cl^- and Ca^{2+} levels [28, 29]. Furthermore, water passively passes the cell membrane following the influx of ions, resulting in brain edema [30].

1.1.4.2. Oxidative stress

Oxidative stress occurs when the generation of free radicals overwhelms the endogenous cellular antioxidant defenses. Because of the low capacity of endogenous antioxidants in neurons [31], the brain is highly vulnerable to reactive oxygen species (ROS). Following brain ischemia, increased levels of intracellular Ca^{2+} , Na^+ and ADP stimulate mitochondria to generate deleterious amount of ROS. Additionally, ischemia also triggers nitric oxide synthase (NOS) activation, which consequently increases the NO production. Interaction of NO and superoxide can yield highly toxic peroxynitrite, a compound that decomposes spontaneously to produce the hydroxyl radical, which is the most reactive oxygen radical and probably causes the most tissue injury [32]. The increase of free radical can induce numerous toxic cellular effects, such as impairing enzyme activity, releasing Ca^{2+} from intracellular stores, damaging cytoskeleton and DNA, protein denaturation, lipid peroxidation and mitochondrial dysfunction [33]. Besides cellular damage, oxidative stress also contributes to the elevation of BBB permeability following brain ischemia through activation of matrix metalloproteinases (e.g. MMPs) [34, 35], as well as direct damage on endothelial cells [36]. Furthermore, the interaction between NO and superoxide induces vasoconstriction hereby reducing the cerebral blood flow.

1.1.4.3. Post-ischemic inflammation

Inflammation associated with cerebral ischemia involves the induction of cytokines, chemokines, and adhesion molecules at the level of brain endothelial cells, in coordination with the trafficking and transmigration of circulating inflammatory cells, as well as the activation of resident brain cells. Continuing amplification of inflammatory responses contributes to severe cellular damage, disruption of microvascular stasis, and impairment of BBB function.

In response to cerebral ischemia, cytokines are upregulated in the brain and expressed not only in cells of the immune system, but also in resident brain cells, including glia, neurons and endothelial cells[37-39]. The widely investigated cytokines related to inflammation in cerebral ischemia are interleukin-1 (IL-1), TNF- α , interleukin-

6 (IL-6), interleukin-10 (IL-10) and transforming growth factor- β (TGF- β). IL-1 and TNF- α are associated with induction of adhesion molecules on endothelial cells during ischemia/reperfusion (I/R) [40]. IL-6 is largely considered to be a proinflammatory cytokine, but whether it plays a significant role in ischemic stroke is far from clear. IL-10 is recognized as an anti-inflammatory cytokine, acting by inhibiting IL-1 and TNF- α and also by suppressing cytokine receptor expression as well as receptor activation. Elevation of proinflammatory cytokines and reduction of the anti-inflammatory IL-10 are related to larger infarctions and poor clinical outcomes [41].

Cytokines also stimulate the production and release of chemokines, which play an important role in cellular communication and inflammatory cell recruitment in the host defense. Expression of chemokines after the onset of cerebral ischemia is thought to be deleterious because of their critical role in leukocyte infiltration[42]. It also suggested that, in addition to chemotactic properties, chemokines can directly affect BBB permeability. For example, exogenous monocyte chemoattractant protein-1 (MCP-1) increased BBB permeability 17-fold *in vitro* and caused alterations in tight junction (TJ) proteins, which indicate that MCP-1 may participate in opening the BBB [43].

Subsequent to the activation of cytokines and chemokines, the induction of adhesion molecules starts. Adhesion molecules are important mediators for leukocytes infiltration into the brain parenchyma. During the early stages of ischemia, E-selectins and P-selectins which mediate the interaction between leukocytes and the vascular endothelium, are upregulated. Once slowed by binding to selectins, contact between leukocyte integrins and endothelial intercellular adhesion molecule-1 (ICAM-1) mediates leukocyte adhesion to and migration through the layer of endothelium, whereas monocytes undergo a similar process by binding to vascular cell adhesion molecule (VCAM) [44]. Inflammatory cells adherence to the cerebral vessel wall or transmigration into the CNS, which are promoted by adhesion molecules, may contribute to the BBB disruption and worsen the stroke outcomes.

Activation of enzyme activity is also involved in the inflammatory cascade. Upon brain ischemia, accumulation of intracellular calcium activates the phospholipase A2, which subsequently triggers the hydrolysis of glycerophospholipids, resulting in releasing

of arachidonic acid (AA). AA metabolites are potent mediators that contribute to post-ischemic inflammation in the brain [45]. AA can be metabolized by two different enzymes, which are cyclooxygenase (COX) or lipoxygenase (LOX). COX-1 is constitutively expressed in microglia, leukocytes and many other cell types during brain injury [46], while upregulation of COX-2 is observed not only in ischemic core, but also penumbra [47]. In addition, COX-2 exerts toxic effects through prostaglandins rather than reactive oxygen radicals [48]. However, the role of the LOX pathway is less clear than the COX pathway in brain ischemia. Leukotrienes (e.g. LTC₄) are metabolites that are generated via LOX pathway. They are chemoattractants and may play role in BBB dysfunction, edema and neuronal death after ischemia/reperfusion [49, 50]. During brain ischemia/reperfusion, biphasic elevation of AA and LTC₄ has been reported and appears to relate to biphasic patterns of BBB disruption[49].

1.2. Blood-brain barrier (BBB)

1.2.1. Overview

The BBB is a highly specialized brain endothelial structure that is localized at the interface between the peripheral circulation and the CNS. The primary responsibilities of the BBB are to ensure constant nutrients supply for the brain and, at the same time, limit paracellular movement of the hydrophilic solutes and trans-endothelial migration of circulating blood cells and pathogens.

1.2.2. BBB in homeostasis

Brain endothelial cells are different from non-brain endothelial cells because of the presence of intercellular tight junctions (TJs). The tightly sealed monolayer of brain endothelium by the TJs primarily confers the low paracellular permeability and high transendothelial electrical resistance (TEER) of the BBB [51]. The maintenance of the TJs is managed by transmembrane proteins, occludin, claudins and junction associated molecules (JAMs), as well as several cytoplasmic proteins, such as zonula occludens

(ZOs) (Fig. 1.1). Occludin is highly expressed in brain endothelial cells and can be consistently detected along the cell borders [52]. Multiple phosphorylation sites, which present on occludin serine and threonine residues, are critical elements for the association of occludin with the cell membrane and the regulation of the BBB permeability [53]. The cytoplasmic C-terminal domain of occludin is associated with accessory proteins, ZO-1 and ZO-2, enabling the connection of occludin with the cytoskeleton [54]. ZO-1 (220 kDa) and ZO-2 (160 kDa) are phosphoproteins, which can form heterodimers with each other. ZOs serve as recognition proteins for TJ placement, and also anchor the transmembrane proteins to the actin cytoskeleton through their proline-rich carboxy-terminus [54]. ZO proteins have also been indicated in recruitment of the transmembrane TJ components to the apical part of the cellular membrane [55]. Claudins, which locate proximately to occludin at the cellular membrane, are a family of more than 24 proteins [56]. The homophilic and/or heterophilic interactions of the claudins formed by the extracellular loops secure the tight connections of the cell monolayers [57]. The contiguous staining of claudins can be detected along endothelial cell borders, which suggests that claudins primarily constitute the TJs, while occludin enhances the tightness further [55]. Junctional adhesion molecules (JAMs) are members of the immunoglobulin super family. JAMs mediate the attachment of adjacent cell membranes via a homophilic or heterophilic interaction [58]. Several JAM proteins have been identified, with JAM-A shown to be significantly expressed on the brain ECs. Studies indicate that homophilic JAM-A interactions are critical in the stabilization of cellular junctions and the reduction of paracellular permeability [59, 60].

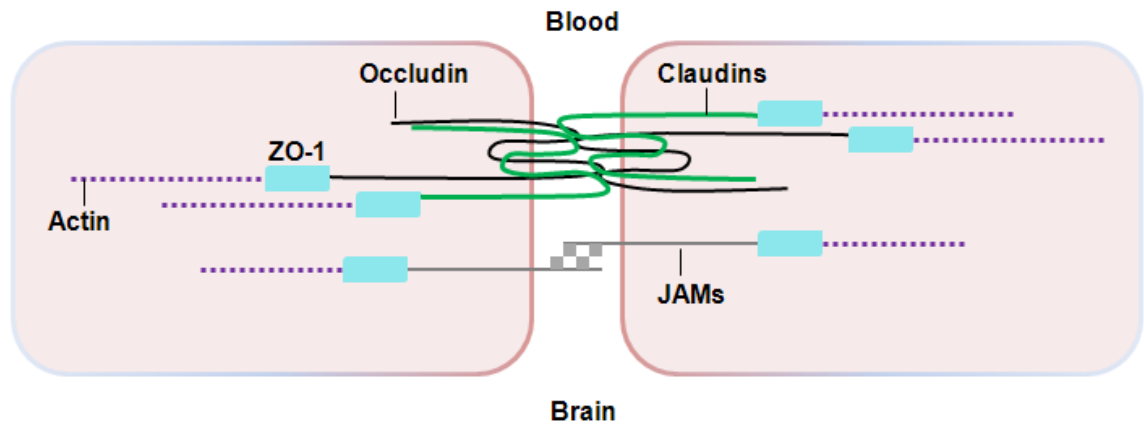


Figure 1.1. The molecular composition of tight junctions (TJs) between two adjacent brain endothelial cells. The TJs consist of transmembrane proteins, including occludin, claudins, and junctional adhesion molecules (JAMs), which are linked to actin cytoskeleton by accessory proteins such as zonula occluden-1 (ZO-1).

Another distinctive character of brain endothelial cells is the polarized expression of membrane receptors and transporters, which can be generally classified into five main categories: carrier-mediated transport, ion transport, active efflux transport, receptor-mediated transport, and caveolae-mediated transport [51]. They control the active transport of nutrients to the brain and the efflux of potentially toxic compounds from the cerebral to the vascular compartment. For example, nutrients such as glucose, nucleosides, purines, amines and vitamins are transported by carrier-mediated transport systems from the blood to the brain [61, 62], while efflux of molecules from the brain endothelium is facilitated by ATP-binding cassette (ABC) transporters [63]. The high density of mitochondria in the brain endothelial cells reflects high energy demands for active ATP-dependent transporters, such as sodium pumps, which regulate the exchange of ions [64]. In addition, receptors for large proteins, such as insulin and transferrin receptors, are found in the caveolar membranes, which are responsible for the transcellular permeability by regulating endocytosis, transcytosis, and signaling in lipid-based microdomains of the BBB [65].

In close proximity to brain endothelial cells, pericytes, glial cells (e.g., astrocytes, and microglia), and neurons, together with the basal lamina form a functional unit, referred to as a neurovascular unit (NVU) (Fig. 1.2). The close interaction of different cell types with each other allows for effective paracrine regulations that are important for maintaining the brain homeostasis as well as modulating the disease processes. Under cerebrovascular disorders, such as stroke, vascular dementia, or hypertension, changes in endothelium and pericytes can directly disturb neuronal and synaptic functions via alteration of the blood flow, BBB permeability, enzymatic functions, secretion of trophic factors and matrix molecules, expression of vascular receptors, or induction of ectoenzymes. In response to a vascular insult, signals from neurons, astrocytes and activated microglia can further amplify the neuronal injury and synaptic dysfunction.

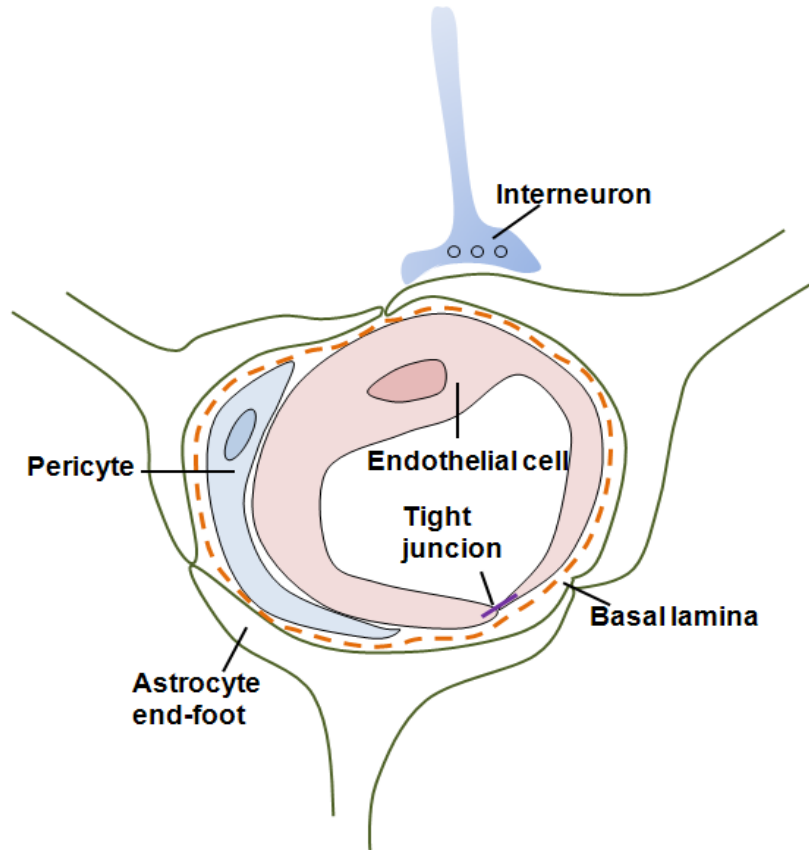


Figure 1.2. Schematic of the neurovascular unit (NVU) of the blood-brain barrier (BBB). The capillary endothelial cells, together with the pericytes, are surrounded by basal lamina and astrocytic perivascular endfeet. Astrocytes also provide the cellular interaction with neurons.

1.2.3. BBB dysfunction in stroke

Under ischemic stroke conditions, BBB dysfunction results in increased paracellular permeability, which directly contributes to cerebral edema, hemorrhagic transformation, and increased mortality. Several mechanisms, occurring in a phasic manner, contribute to the ischemic damage of the BBB. As soon as 2 hours after the onset of ischemia, dissolution of the endothelial basal lamina starts. This is rapidly followed by an increase in BBB permeability. Upon reperfusion, a biphasic increase of BBB permeability may occur. The multi-phasic phenomenon of this permeability change is determined by various factors, including the duration of ischemia, degree of reperfusion, and the experimental models of stroke that are used for assessment. The increase of paracellular permeability is generally associated with the alteration of TJ protein expression and/or the redistribution of TJ protein along the cellular membrane. It has been shown that a reduction in TEER is accompanied by a decreased claudin-5 expression under the hypoxic conditions [66], whereas hypoxia induced increase of paracellular permeability has been observed along with the disruption of occludin, ZO-1, and ZO-2 membrane localization [67]. In response to brain ischemia, accumulated bradykinin, vascular endothelial growth factors, and thrombin initiate the increase of intracellular calcium concentration, whose principle effect in the endothelial cells is the activation of calcium/calmodulin-dependent myosin light chain kinase (MLCK) [68], inducing actin reorganization, changes of cell shape, and thus increased BBB paracellular permeability [69]. Oxidative stress is also an early stimulus for BBB disruption and triggers the cellular release of MMP-9 from neurons, astrocytes, pericytes, and endothelial cells, which results in digestion of the endothelial basal lamina. In the later phase, severe BBB damage resulting from more complicated mechanisms appears, such as induction of proinflammatory cytokines, followed by chemokines and adhesion molecules expression on the activated endothelium [70]. The expression of cytokines and adhesion molecules precedes the infiltration of leukocytes, which, together with activated microglia, further enhances the inflammatory responses and the production of toxic free radicals [70, 71]. The damaged BBB allows leakage of blood constituents into the brain parenchyma. Extravasation of high molecular weight components, which is followed by water due to osmosis, concomitantly leads to vasogenic edema and intracranial

hypertension. Particularly, extravasation of red blood cells may lead to hemorrhagic transformation in the injured brain.

1.3. Telomere and telomerase

1.3.1. Overview

Telomeres are specialized DNA structures localized at the ends of chromosomes and primarily function to maintain the genome stability. Telomeres consist of non-coding tandem repeats of the TTAGGG sequences and telomere-specific proteins [72]. Because of the complications exhibited during DNA replication called-- the end replication problem, telomeres continuously lose the guanine-rich repeats in a manner that is coupled with cell division [73]. The shortening of telomere is prevented by telomerase, an enzyme which can synthesize telomeric DNA and maintain the function of telomere [74]. Human telomerase is composed of two core components: telomerase RNA component (TERC) that serves as a template, and telomerase reverse transcriptase (TERT), which is a catalytic subunit of telomerase [75]. The enzyme activity of telomerase is detectable in early fetal development, but is repressed in most of the postnatal somatic tissues [72, 76]. In proliferating cells without functional telomerase, telomeres gradually erode with time and mitotic division, and the cells eventually undergo senescence. Although telomerase repression has been extensively studied as a mechanism for tumor suppressor in cancer research, increasing evidence from epidemiology and laboratory data indicates that shortening of telomere and repression of telomerase are involved in a broad spectrum of human diseases. For example, patients with Dyskeratosis congenita, a congenital disorder, are diagnosed with shortened telomeres because of the telomerase mutation. In addition, telomere length shortening has been recognized as a marker of biological aging and may also play an important role in age-related diseases, such as cardiovascular diseases and stroke [77]. Individuals with a telomere length shorter than the average are at higher risk of heart disease or stroke; on the other hand, it has also been revealed that telomere shortening in humans suffering from long-term chronic stress is more accelerated as compared to the people of a similar age [78-81]. The laboratory evidence suggested that

telomerase transduction in the cells can extend the replication capacities, protect cells from stress, and improve functional activities while the differentiation and growth of the cells are still under control [77]. Thus, telomerase may be a novel target for the treatment of diseases associated with telomere loss and aging.

1.3.2. The roles of telomerase reverse transcriptase (TERT) in stroke

In the developing brain, TERT expression can be detected in neuronal progenitor cells, embryonic neurons and early postnatal neurons; however, TERT level declines progressively after birth [82-84]. Recently, studies revealed that TERT expression is inducible in the damaged brain cells of adult animals subjected to the occlusion of middle cerebral artery, indicating an important role of TERT in ischemic brain injury. Additionally, under pathophysiological conditions, such as ischemic stroke, TERT has been reported to reduce the excitotoxicity, promote neuronal survival and differentiation, and increase angiogenesis in the CNS [85, 86].

As mentioned before, excitotoxicity, which is mediated by over-stimulation of NMDA receptors, is one of the main mechanisms to induce neuronal death following brain ischemic injury. A recent study reported that injection of a lethal dosage of NMDA accelerated the death of TERT-deficient mice, while transgenic mice with overexpression of TERT showed an improved survival rate as compared to wild-type littermates [87]. In addition, it was demonstrated that transgenic mice overexpressing TERT showed significantly decreased infarct volume after permanent occlusion of the middle cerebral artery. The reduced brain injury is associated with the defensive role of TERT, whose overexpression protects against NMDA receptor-induced excitotoxicity through decreasing cytosolic accumulation of Ca^{2+} while enhancing Ca^{2+} uptake by mitochondria [86]. These results suggest that TERT may protect NMDA-mediated excitotoxicity through a mitochondrial related signaling pathway.

The anti-apoptotic effect of TERT has been observed in various cell types including neuronal cells [88-90]. Subsequently, it has been suggested that TERT can protect neurons against apoptosis induced by DNA damage [84], oxidative stress and

ischemia [86]. Because of the reverse transcriptase activity of TERT, the anti-apoptosis effect of TERT may work through the maintaining of the chromosome integrity and preventing DNA damage, an event which is also involved in the apoptosis induced by ischemic injury [91]. Additionally, studies also reported that TERT can suppress apoptosis induced by dysfunction of mitochondria following cerebral ischemia [86, 87]. TERT is normally localized in the nucleus; however, the export of TERT from the nucleus to the mitochondrial matrix has been detected under stressed conditions, indicating an important role of TERT on mitochondria [92, 93]. It has been shown that TERT can suppress apoptosis before the release of cytochrome c and apoptosis-inducing factors from mitochondria. Further studies suggested that TERT regulates apoptosis by interacting with the Bcl-2 family [94, 95]. In addition, accumulating research reported that TERT can maintain the function of mitochondria and thus suppress apoptosis by protecting mitochondrial DNA (mtDNA), reducing the production of ROS, increasing the mitochondrial membrane potential, and facilitating mitochondrial coupling [92, 93, 96]. Thus, the role of TERT in mitochondria-mediated apoptosis may also account for its protective effect in cerebral ischemic injury.

Angiogenesis plays a vital role in the repair mechanisms following a brain ischemic injury. It has been shown that sustained TERT activity contributes to the angiogenic properties of cultured endothelial progenitor cells [97]. Additionally, *in vivo* studies reported that TERT significantly induced new capillary formation in ischemic tissue. The underlying neo-angiogenic mechanisms of this study include VEGF-mediated induction of telomerase enzyme activity and expression of TERT through the nitric oxide signaling pathway, and telomerase-dependent endothelial cell differentiation and anti-apoptosis [98]. On the other hand, down-regulation of TERT results in decreased neovascularization, which is associated with reduced cell proliferation and expression of key molecules involved in angiogenesis, such as VEGF [99]. Furthermore, it has been reported that TERT knockdown *in vivo* leads to suppression of genes related to anti-apoptosis and angiogenesis.

1.4. Polychlorinated biphenyls (PCBs)

1.4.1. Overview

PCBs are known as chlorinated hydrocarbons and were first synthesized in 1881. They are oily, light colored liquids with special physical properties including high flash points, chemical stability, and low electrical conductivity. Due to these characteristics, PCBs were widely applied to lots of industrial and commercial purposes, such as pigments, transformers, and rubber products. Industrial or commercial PCBs used in those products were chemical mixtures which consisted of different chlorinated biphenyls components. In the United States, most of the commercial PCB mixtures are recognized by their trade name—Aroclor, the most commonly known PCB mixture. Because of a wide range of toxic effects of PCBs, they were banned by the EPA in 1979, and ultimately out of use in 1983. However, PCBs can still be identified in approximately one-third of the sites listed on the National Priorities List (NPL), a list associated with the Superfund Program [100]. PCBs are resistant to natural chemical and biological processes, thus, banned PCB production does not imply reduced toxicity. In many parts of the world, wide distribution of PCBs in the environment and their adverse health effects on humans and animals have already raised the concern of investigators and researchers.

There are several sources of human exposure to PCBs, including food, drinking water and air. Oral exposure to PCBs is the predominant route because PCB congeners are lipophilic and they can bioaccumulate in the food chain. It is currently assumed that the general population is exposed to PCBs mainly through food intake, including ingestion of fatty fish, beef, dairy products, and also postnatal breast milk. Two well-known examples of accidental intake of highly PCB contaminated rice oil are Yusho in Japan in 1968 and Yu-Cheng in Taiwan in 1979 [101]. Other populations that may have higher PCB contamination include traditionally fish-eating communities [102-104]. People who work in PCB-disposal facilities or PCB-contaminated buildings, as well as those living in close proximity to former PCB-manufacturing plants, such as the Monsanto plant in Anniston, AL, and on the upper Hudson River, NY may also be subject to high exposures [105, 106]. In addition, infants are at higher risk of exposure than adults because PCBs bioaccumulate in milk.

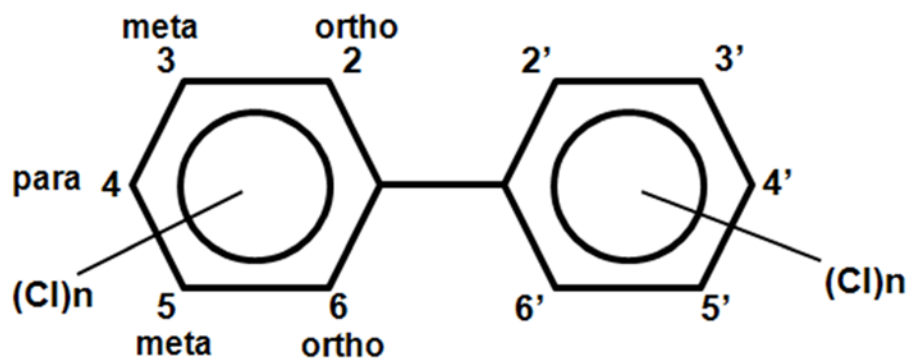
1.4.2. Mechanisms of PCB toxicity

PCBs have been demonstrated to cause diverse and severe health effects. Theoretically, PCBs consist of 209 congeners, of which only about 130 congeners have been identified in commercial products [107] (Fig. 1.3A). Each of the congeners has a different toxic level and health effect on the human body. The toxic diversity complicates exposure assessment and reduces the validity of statements made about PCBs in general. Animal studies revealed that the toxic effects of PCBs can result in cancer and a number of non-cancer problems, such as the effects on disruption of the immune system, reproductive system, nervous system, and endocrine system [108]. The various health effects caused by PCBs may be dependent on each other in such a way that interruption in one of those systems by PCBs may extensively relate to other regulatory systems of human body.

Different PCB congeners exhibit their toxicity in different manners [109]. PCBs can be categorized into different groups according to their inductive effects to various cytochrome P450 (CYP) isoenzymes. The pattern of CYP enzyme induction is largely dependent on the degree of planarity of the biphenyl rings, which is determined by the position of the chlorines, especially by the number of ortho chlorines that generate steric force to the rotation of the rings. The toxicity of coplanar PCB congeners depends on the activation of an intracellular receptor known as aryl hydrocarbon receptor (AhR). AhR is a ligand-activated transcription factor that belongs to the steroid-hormone receptor family. However, AhR has no recognized endogenous ligand, but only exogenous aromatic compounds such as certain polycyclic aromatic compounds and halogenated aromatic compounds like the dioxins. Activation of AhR by coplanar PCBs such as PCB77, PCB126, and PCB 169 results in the expression of cytochrome P450 1A1, 1A2, 2A1, and 1B1 genes. Expression of these genes has been demonstrated to result in the proliferation of endoplasmic reticulum in the liver; this leads to the alteration of liver functions in many aspects, including the perturbation of endocrine function. The second group of PCBs consisting of the mono-ortho and some di-ortho components, so called “mixed” congeners, keeps most of the coplanar feature of the rings, which enables binding to the AhR with intermediate affinity and induces the enzyme expression of CYP 1A, CYP 1B, and CYP 2B. PCB105, PCB118, PCB138, and PCB 156 belong to this

group. The third category is composed of congeners having *di-ortho* chlorines with one or more *ortho* positions chlorinated on both phenyl rings, for example , PCB99 and PCB153 (Fig 1.3B). These are non-coplanar PCB congeners, which barely have binding affinity to the AhR and preferentially induce the expression of CYP 2B instead of CYP 1A. PCB congeners in this group show a variety of toxic effects, including carcinogenesis and neurotoxicity, which are different from that of the coplanar PCBs [110]. Additionally, some *ortho*-substituted congeners without AhR binding activity have a totally different profile of toxic effects that are unrelated to any CYP enzymes. Several studies reported that such PCBs can change the homeostasis of the cells of the nervous [111-113] and immune systems [114, 115], and cause a relatively rapid cell death resulting from disruption of the membrane structure, an effect which has not been detected in coplanar PCBs at similar concentrations. These PCB congeners can also disrupt the release of insulin, decrease the synthesis of dopamine, and activate neurophils to produce ROS in neurons [116].

A



B

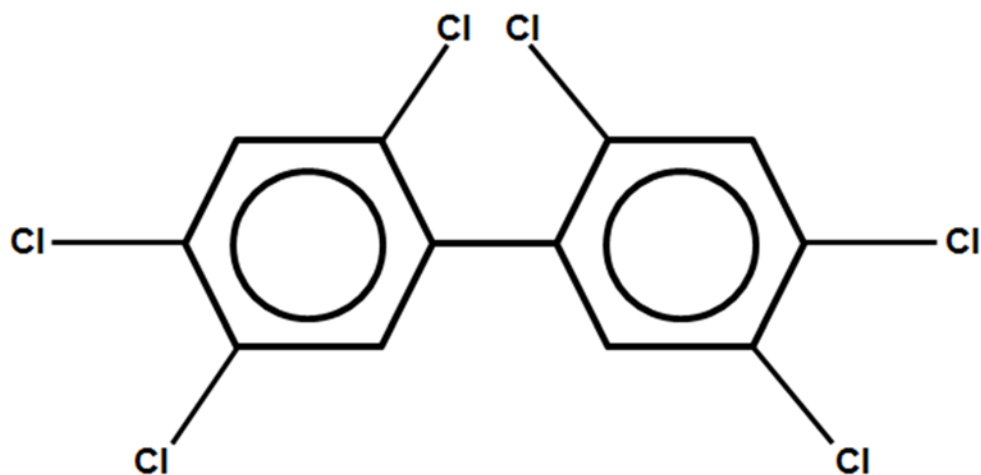


Figure 1.3. Chemical structure of the PCB molecule. (A) A biphenyl molecule showing chlorine substitution ($n=0-5$). (B) A specific structure of PCB153 (2,2',4,4',5,5'-hexachlorobiphenyl).

1.4.3. Neurotoxicity of PCBs

Exposure to PCBs may result in impairment of both adult neurologic functions and early childhood development. For instance, epidemiological and clinical evaluations of individuals in the Yusho and Yu Cheng incidents indicate the potential neurotoxic effects of PCBs [117]. Exposure to PCBs during the early development of children is also linked to decrements in learning [118-121]. On the other hand, experimental studies indicate that PCBs have neurotoxic effects on rodents and primates. In laboratory rodents, the most significant neurological effects of exposure to PCBs have been detected as changes in locomotor activity [122, 123] and altered dopamine function [124, 125]. Recent studies demonstrated that exposure to PCBs is associated with central nervous system disease, such as Parkinson's disease (PD), amyotrophic lateral sclerosis, non-Alzheimer-related dementia, and brain cancer in adults [126-128]. Exposure to PCBs is also considered as one of the risk factors for a stroke. For example, recent epidemiologic and laboratory data suggest that exposure to PCBs is associated with increased risk factors for stroke, such as diabetes, hypertension and vascular disease [108, 129-131]. Specifically, it has been demonstrated in the residents in Anniston, AL, that there is a significant association between serum PCB concentrations and the prevalence of hypertension. [132]. In addition, it has been reported that living close to hazardous waste sites contaminated with PCBs is highly linked to increased stroke incidence [14]. However, so far there is a general lack of studies to address the influence of PCBs on the stroke outcomes and the mechanisms involved in PCB-induced stroke.

The neurotoxicity of PCBs is likely due to their ability of accumulating in the brain tissue. Since PCBs are highly lipophilic, they tend to accumulate in the lipid-rich tissue, such as brains, which contain high lipid content. The highest level of PCBs in the brain was detected in dead polar sea gulls from the islands of Svalbard, which ranges from 3 $\mu\text{mol/kg}$ to 90 $\mu\text{mol/kg}$ [133]; whereas, the PCB brain level in the live glaucous gulls was 1.5–30 $\mu\text{mol/kg}$ [134]. In experimental conditions, chronic exposure to PCB mixture [126] and acute exposure to individual PCB congeners [135] both resulted in detectable PCB levels in animal brains.

Although the mechanisms have not been clearly elucidated, endothelial activation has been implicated as a result of PCB-induced vascular dysfunction [136, 137]. The

vascular endothelial cells, which function as a selective barrier between blood and the vessel walls, may be targeted by various environmental toxicants, such as PCBs [138]. The most widely studied mechanism of PCB-induced endothelial dysfunction was focusing on coplanar PCBs [136], because the AhR signaling pathway is also expressed in the vasculature [139], and endothelial cells show an induction of CYP1A1 following exposure to AhR agonists [140]. Later it was reported that *ortho*-substituted non-coplanar PCBs, for example, PCB104, can also induce endothelial toxicity [141, 142]. Since the BBB is mainly composed of endothelial cells, the dysfunction of brain endothelium may impair the intact brain environment, and thus influence the neuronal homeostasis and induce neurotoxicity. Results published in our laboratory suggested that PCB153, the most abundant non-coplanar PCB congener in the environment, can activate the human brain endothelial cells by over expression of VCAM-1 and ICAM-1 through NADPH oxidase and lipid raft-associated redox signaling pathway [143]. Furthermore, we also demonstrated a differentially altered expression of the TJ proteins in brain capillaries as well as disrupted function of blood-brain barrier in mice that were acutely exposed to three different PCB congeners, including coplanar PCB126, mono-*ortho*-substituted PCB118, and non-coplanar PCB153 [144].

1.5. Nanoparticles

1.5.1. Overview

Nanosized particles exist widely in the environment, for example, the products of photochemical and volcanic activity. Humans have experienced the exposure to nanosized particles throughout their evolutionary phases; however, such exposure has increased dramatically in the past century because of anthropogenic causes, such as thermo-degradation, internal combustion engines, and power plants. In addition, due to the rapidly development of nanotechnology, engineered nanoparticles are likely to become another source for human exposure.

Ambient particulate matter (PM) can be characterized into three categories according to their sizes: coarse particles with an aerodynamic diameter in between 2.5

μm and $10\ \mu\text{m}$, fine particles with the diameter of less than $2.5\ \mu\text{m}$, and ultrafine particles (UFPs) of less than $100\ \text{nm}$ [145]. Although UFPs are particles in the same size range as engineered nanoparticles, they come from different origins and are either produced from everyday life, or released as industrial byproducts. Thus, nanometer-scaled particles can be roughly divided into three categories: 1) naturally occurring UFPs; 2) inadvertently produced by human activity, which also can be named as anthropogenic UFPs; and 3) engineered nanoparticles that are deliberately produced for specific purposes [146].

1.5.2. Applications of nanotechnology

According to the United States Nanotechnology Initiative, nanotechnology is “the understanding and control of matter at dimensions of roughly $1\text{--}100$ nanometers, where unique phenomena enable novel applications.” Indeed, nanotechnology has been increasingly applied to industrial purposes in the last decade, including cosmetics, electronics, aerospace and computer industry. Additionally, given the pressing needs of developing new medicines, engineered nanoparticles have been used in medical fields, such as biomarkers, diagnostics, and drug delivery.

1.5.3. Toxicity of nanoparticles

The key properties that have emerged as determinants of cellular uptake, subcellular localization, and cytotoxicity of nanoparticles include size, shape, chemical composition and surface modification. It has been shown that a significant amount of nanoparticles ($1\text{--}100\ \text{nm}$) are deposited in regions of the respiratory tract, with $20\ \text{nm}$ nanoparticles having the highest deposition efficiency ($\sim 50\%$) in the region of alveolar [147]. In addition to the particle size, the surface characteristics of the nanoparticles can result in specific binding interactions, such as receptor-mediated endocytosis, as well as nonspecific binding interactions with the cellular membrane [148]. Once deposited, nanoparticles tend to translocate to the extrapulmonary sites, circulate with the blood and reach other organs through various mechanisms. One of the well-recognized mechanisms

involves transcytosis of nanoparticles, which cross epithelia of the respiratory tract into the gastrointestinal tract and access the blood circulation directly or via lymphatics, resulting in distribution of nanoparticles throughout the body. Deposited nanoparticles have also been shown to reach the CNS through the sensory nerves that are embedded in the airway epithelia [149] and/or BBB [150, 151]. Studies focusing on nanotoxicity suggest that nanoparticles are capable of entering the human body and exhibiting toxic effects at the cellular levels [152, 153]. Different surface coatings of nanoparticles lead to different cellular toxic levels. For instance, cationic polystyrene nanospheres increase the cytotoxicity in vitro and pulmonary toxicity in vivo as compared with anionic and neutral ones [154]. Specific cytotoxicity of silica, which is generally accepted as inert nanomaterial, is strongly associated with the presence of surface oxygen radicals and ROS [155]. Because of the small size and high surface area of nanoparticles, they can easily bind to and hence facilitate the transport of environmental pollutants, which may result in efficient delivery of these toxicants into the human body. Air born particles, usually considered as efficient carriers for various pollutants from the ambient air, have been linked to asthma and cardiovascular dysfunction [156], as well as neurodegenerative diseases [157].

1.5.3.1. Cardiopulmonary toxicity of nanoparticles

One of the major target organs of nanoparticles and ambient PM is the lung. The special characteristics of fine and ultrafine particles, including size, surface area, and chemical composition, are all related to the health risks of exposure to PM. It has already been reported that elevated ambient air pollution is associated with adverse health effects in children, vulnerable adults and patients with asthma [158]. According to recent studies, exposure to ambient air pollution can cause decreased lung function, increased respiratory infections, and chronic obstructive pulmonary disease (COPD), resulting in increased hospitalization and even mortality [159-162].

On the other hand, increased cardiovascular mortality caused by ambient air pollution has also been reported in numerous epidemiologic investigations [163, 164]. In

addition to the epidemiology studies, the pathophysiologic evidence documented the relationship between particulate air pollution and cardiovascular diseases, such as pulmonary and systemic inflammation, accelerated atherosclerosis, and altered cardiac autonomic function [165]. A 16-year follow-up study of 500,000 adults reported that every 10 $\mu\text{g}/\text{m}^3$ elevation of PM_{2.5} was linked to an 8–18% increase in mortality, which resulted from ischemic heart disease, dysrhythmias, heart failure, and cardiac arrest [166].

There are three mechanisms that have been proposed to explicate the cardiopulmonary morbidity and mortality caused by fine and ultrafine PM [156]. The first hypothesis is that the ultrafine or fine PM reaching the lung can stimulate the neurons in the lung, influencing CNS and cardiovascular autonomic function. The second hypothesizes that fine PM can enter directly into the systemic circulation and translocate to target organs, thus initiating inflammatory responses, secreting cytokines, reactive oxygen species (ROS), and C-reactive proteins, thereby inducing cardiac dysfunctions. The third mechanism proposes that fine PM induces lung injury via oxidative stress, which results in increased synthesis of proinflammatory proteins. These events then trigger the activation of mitogen-activating protein kinase (MAPK), redox-sensitive transcription factors, nuclear factor kappa B (NF- κ B), and activating protein-1 (AP-1), thereby further enhancing the inflammation in the lung, and leading to cardiac events. Inflammation is strongly associated with atherosclerosis. In fact, it has already been reported that genetically susceptible mice exposed to ambient air PM chronically exhibit acceleration of vascular inflammation and atherosclerosis [167]. Furthermore, human studies also support the link of inflammation-induced developments of atherosclerosis after exposure to ambient air PM [168].

1.5.3.2. Neurotoxicity of nanoparticles

It is estimated that in the United States 29 million people are exposed to PM₁₀, and 88 million are exposed to PM_{2.5}; whereas, exposure level of UFPs, although estimated to be high, are unmonitored and unregulated in the U.S. It is well-recognized that PM can induce adverse health effects through the cardiovascular and respiratory

systems, while their deleterious consequences on human brains have only lately been investigated. PM has been suggested to potentially affect CNS diseases, including Alzheimer's disease (AD), PD, and stroke. A recent study reported that individuals living in highly polluted areas showed elevated proinflammatory responses in the brain, endothelial cell activation, BBB damage, and brain lesions. More important, increased amyloid beta deposition, a hallmark protein of AD, was also detected in the brain tissue from studied individuals [169]. In addition, although data is limited, exposure to different air pollutants, including PM, is epidemiologically related to increased risk for cerebral ischemic events [148, 154, 170] .

As mentioned above, nano-sized particles can translocate to the brain via olfactory nerves and/or BBB. It has been demonstrated in animal models that intranasally instilled or inhaled nanoparticles are able to migrate to the olfactory nerves and olfactory bulb, or are taken up into the brain directly through trans-synaptic transport [171]. The transportation of nanoparticles across the BBB is also possible, which can be mediated either by passive diffusion or endocytosis [155]. The ability of nanoparticles to cross the BBB and accumulate in a different region of brain [172] may be beneficial for drug delivery; however, it also raises the concern of cellular toxic effects that are imposed by these nanoparticles. For instance, data published recently suggest that exposure to nanoparticles results in damage on neurons [173] and microglia [174], CNS oxidative stress [175], neuroinflammation [176], BBB alterations, and cerebrovascular damage [145]. The interactions between nanoparticles and/or PM and brain endothelium have also been investigated. Results published from our laboratory show that exposure to manufactured alumina nanoparticles induces damage on human microvascular endothelial cells and BBB disruption through alteration of mitochondrial potential, elevation of oxidative stress, and decrease of TJ protein expression [177]. Additionally, one study using capillaries extracted from rat brains reported that exposure to particulate matter can cause increased generation of ROS and cytokines, which subsequently changes the expression and function of transporters, such as P-glycoprotein and Multidrug Resistance Associated Protein-2 [178]. More important, ultrafine PM deposition was observed in the human brain after long-term exposure to air pollution, which is also associated with endothelial cell damage and increase in ICAM and VCAM

expression in cerebral vasculature [169]. Therefore, cerebral capillaries may recognize and respond to nano-sized particles by producing proinflammatory signals, modulating oxidative stress, and regulating physical and chemical barrier function, ultimately leading to nanoparticles accessing the brain parenchyma and further contributing to CNS pathology.

1.6. Toll-like receptor 4 (TLR4)

1.6.1. Overview

Toll-like receptors (TLRs) are pattern-recognition receptors, which play a pivotal role in the innate immune response by recognizing and responding to invading pathogens [179]. To date, 11 human and 13 mouse TLRs have been identified in both immune and non-immune cells [180]. TLRs can be categorized into 3 types according to the specific pathogen-associated molecular patterns (PAMPs) they recognize. The first group recognizing forms of lipids includes TLR1, TLR2, TLR4 and TLR6. The second group of TLRs, which are activated in response to protein ligation, is composed of TLR5 and TLR11. TLRs in the third group are localized in the endosomes, an ideal position where nucleic acids of bacterial and viral origin can easily access to. This group consists of TLR3, TLR7, TLR8 and TLR9. However, the ligands for TLR10, TLR12 and TLR13 have not been identified yet [180]. In recent years, a growing body of evidence indicates that TLRs may recognize endogenous ligands that are released from damaged tissues following ischemia/reperfusion injuries, such as those in stroke [181], and mediate the immunologic response in noninfectious cells. During stroke-induced tissue injury, components of extracellular matrix undergo cleavage by enzymes such as MMPs, revealing moieties that may activate TLRs and then initiate TLR-mediated signal transduction. The endogenous ligands of TLRs include fibrinogen [182], fibronectin [183], low-molecular-weight hyaluronic acid (HA) [184], and heparin sulfate proteoglycans [185].

Upon activation, TLRs can form either homodimers or heterodimers with other TLRs and initiate the signaling pathway by recruiting numerous accessory proteins. TLR-

activated signaling pathways are generally classified into two categories: myeloid differentiation factor 88 (MyD88)-dependent and -independent pathways. Recruitment of MyD88 is the universal event of all TLR activation, except for TLR3 [186, 187].

1.6.2. Toll-like receptor 4 (TLR4) signaling

It is well-known that the exogenous ligand of TLR4 is endotoxin such as lipopolysaccharide (LPS), which is an outer membrane component of gram-negative bacteria [188]. The activation of TLR4 is complex and involves two principle auxiliary proteins, LPS-binding protein (LBP) and cluster differentiation antigen 14 (CD14), as well as a coreceptor, myeloid differentiation protein (MD-2) [189]. It has been described that MD-2, but not TLR4, directly binds to the conserved component of endotoxin -- amphipathic lipid A [190, 191]. The interaction between the acyl chains of lipid A and MD-2 is crucial for the heterodimerization of MD-2 and TLR4, which is required for the activation of TLR4 signaling cascade [192, 193]. Among all the TLRs, TLR4 is the only one that is capable of signaling through both MyD88-dependent and -independent pathways [179, 194]. After TLR4 activation, MyD88-dependent pathway induces production of proinflammatory cytokines, including TNF α and IL-1 β via activation of NF- κ B; while MyD88-independent pathway culminates in interferon activation via IRF3 (interferon regulatory factor) [195]. MAPK signaling can be induced in both MyD88-dependent and independent pathways, leading to phosphorylation and activation of the transcription factor AP-1 [196]. The activation of TLR4 provokes fast and robust host immune responses. Endotoxin recognition by TLR4 can initiate a rapid production of cytokines, which facilitates the recruitment of inflammatory cells to the infection site [197].

1.6.3. TLR4 and stroke

The role of TLR4 in stroke has been increasingly recognized. For example, mRNA level of TLR4 was upregulated following cerebral ischemia in mouse neurons [198, 199]. Furthermore, the brain edema and the infarct volume in mice with TLR4 deficiency were reduced as compared to wild-type animals after cerebral focal ischemia

injury. In addition, TLR4-deficient mice subjected to experimental stroke models exhibited improved neurological behavior and decreased levels of proinflammatory cytokines in the serum [199-201]. Results from a recent study indicated that the lower infarct volume and moderate neurological deficits of TLR4-deficient mice are associated with reduced expression of deleterious mediators, such as iNOS and COX2, that induced by stroke. Additionally, the expression of MMP9, which plays a role in the cleavage of extracellular matrix and contributes to brain damage, was also decreased in TLR4-deficient animals following cerebral ischemia [202, 203]. Altogether, these studies indicate the importance of TLR4 signaling in modulating the outcomes of ischemia-induced brain damage and suggest that TLR4 may be a target for ischemic stroke therapy in the future.

1.6.4. TLR4 and environmental pollutants

TLR4 is considered as a candidate involved in the susceptibility of toxicity of air pollution. For instance, a genomewide linkage analysis identified TLR4 as one of the candidate genes for ozone-induced hyperpermeability [204]. A study showed that exposure to ozone increased the expression of TLR4 on lung macrophages; additionally, TLR4-deficient mice were partially protected from airway hyperresponsiveness (AHR) following ozone exposure [205]. In addition, chronic exposure to fine PM induced a systemic inflammatory response via TLR4/NADPH oxidase-dependent mechanisms [206]. It has also been suggested that cigarette smoke can activate the intracellular molecules, including MAPKs, IL-8, and MMP-1, via TLR4 signaling pathway [207]. Therefore, it appears that in addition to endotoxin, other environmental pollutants may also have a potential to activate TLR4 signaling through a non-classical TLR4 recognition.

2. General hypothesis and specific aims

Disruption of the BBB is associated with a number of acute and chronic disorders of the CNS, such as stroke. Additionally, the integrity of BBB can be affected by various endogenous and exogenous factors.

With the knowledge that individuals with short telomeres are prone to the development of neurodegenerative diseases, our first hypothesis of this dissertation is that the modulation of telomerase enzyme activity can influence the progression of stroke outcomes. To study this hypothesis, the following specific aim was proposed:

Specific aim 1: To study the hypothesis that deficiency of telomerase activity aggravates stroke outcomes through disruption of the blood–brain barrier and potentiation of neuroinflammatory responses.

PCBs and nanoparticles are external factors which contribute to the modification of BBB function. However, the cerebrovascular toxicity of PCBs assembled onto nanoparticles is unknown. We have selected PCB153 as an example of highly chlorinated non-coplanar PCBs and hypothesized that exposure to PCB153 assembled onto nanoparticles contributes to the development of stroke by disruption of the integrity of the cerebral endothelium through stimulation of toll-like receptor 4 (TLR4) and induction of proinflammatory signaling pathways. To test this hypothesis, two specific aims were proposed:

Specific aim 2: To study the hypothesis that binding PCB153 to nanoparticles potentiates vascular toxicity and brain damage in an experimental stroke model.

Specific aim 3: To study the hypothesis that enhanced brain injury induced by PCB153 bound to nanoparticles is mediated by activation of TLR4, stimulation of inflammatory responses, and alterations of tight junction protein expression in brain capillaries.

Chapter two: Deficiency of telomerase activity aggravates the blood-brain barrier disruption and neuroinflammatory responses in a model of experimental stroke

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1. Synopsis

Epidemiology and genetic studies indicate that patients with telomere length shorter than average are at higher risk of dying from heart disease or stroke. Telomeres are located at the ends of eukaryotic chromosomes which demonstrate progressive length reduction in most somatic cells during aging. The enzyme telomerase can compensate for telomere loss during cell replication. The present study is aimed to investigate the contribution of telomerase to stroke and the blood-brain barrier (BBB) dysfunction. Telomerase reverse transcriptase knock-out (TERT $-/-$) mice and littermate controls with normal TERT expression were subjected to a 24 h permanent middle cerebral artery occlusion (pMCAO). The stroke outcomes were assessed in terms of neurological scores and infarct volumes. In addition, we evaluated oxidative stress, permeability across the BBB, and the integrity of tight junctions in brain microvessels. Neurological testing revealed that TERT $-/-$ mice showed enhanced deficits as compared to controls. These changes were associated with a greater infarct volume. The expression of tight junction protein ZO-1 decreased markedly in ischemic hemispheres of TERT $-/-$ mice. The brain microvessels of TERT $-/-$ mice also were more susceptible to oxidative stress, revealing higher superoxide and lower glutathione levels as compared to mice with normal TERT expression. Importantly, TERT deficiency potentiated the production of inflammatory mediators, such as TNF- α , IL-1 β and ICAM-1 in the ischemic hemispheres of mice with pMCAO. Our study suggests that TERT deficiency can predispose to the development of stroke in an experimental model of this disease.

2. Introduction

Telomeres are located at the ends of eukaryotic chromosomes and protect these regions from degradation. Telomerase reverse transcriptase (TERT) is the catalytic subunit of telomerase that is required for the synthesis of new telomeric DNA repeats in dividing cells [208]. Telomerase activity is down-regulated before birth and remains at very low or undetectable levels in most human somatic tissues [77]. However, a growing body of evidence suggests that TERT expression in adult brain may be induced as the result of metabolic stress, such as hypoxia or brain ischemia, in astrocytes, microglia, vascular smooth muscles, and cortical neurons [86, 209-211]. For example, TERT transgenic mice show a reduced myocardial infarct area after coronary artery ligation, and exogenous TERT expression in cardiac myocytes can prevent apoptosis and promote survival [212].

The blood-brain barrier (BBB) is a physical and metabolic barrier separating the microenvironment of the central nervous system (CNS) from the peripheral circulation. The BBB is mainly composed of brain endothelial cells connected by tight junctions (TJs), which are maintained by transmembrane TJ proteins, such as occludin, claudins and junctional adhesion molecules (JAMs) [213]. Moreover, cytoplasmic scaffolding proteins, such as zonula occludens (ZO) proteins, anchor the integral transmembrane proteins to actin cytoskeleton. Under ischemic stroke conditions, depletion of blood flow leads to the BBB disruption and increased paracellular permeability across cerebral vessels [214]. Activated brain microvascular endothelium in response to environmental stress can exhibit increased cellular oxidative stress, activation of redox-sensitive transcription factors, and production of inflammatory molecules [215]. Ischemia is a potent inducer of cytokines, such as interleukin-1 β (IL-1 β), tumor necrosis factor- α (TNF- α), and adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1).

The function of telomerase in the injured and ischemic brain has not been clearly defined. Therefore, the aim of our present study is to explore the influence of TERT deletion on the BBB integrity and brain inflammatory responses in the context of ischemic brain injury. Our study provides evidence that TERT deficiency potentiates

ischemia-induced neurological deficits, tissue injury, and BBB dysfunction as compared to control mice with normal TERT expression.

3. Materials and methods

TERT deficient mice and the model of the permanent middle cerebral artery occlusion (pMCAO)

All experiments were performed following the protocol approved by the Institutional Animal Care and Use Committee in strict accordance with the National Institutes of Health guidelines. The focal ischemia model employed in the present study was based on a permanent occlusion of the middle cerebral artery (MCA) as described earlier [216]. Sham animals were operated upon with only ligation of the left CCA, without blocking the MCA. The severity of neurological deficit was evaluated according to the modified scale by Wauquier et al [217] (Table 2.1).

The experimental animals (4-month-old male mice) were divided into four groups: wild-type mice subjected to pMCAO, wild-type sham-operated mice; TERT $-/-$ mice subjected to pMCAO, and TERT $-/-$ sham-operated mice. TERT $-/-$ mice were generated on the C57BL/6J genetic background and bred through heterozygous mating [218]. Littermate mice with normal TERT expression were used as the control group.

Evaluation of cerebral infarct volume

The infarct volume was assessed 24 h post induction of pMCAO using staining with 2% 2,3,5-triphenyltetrazolium chloride (TTC) (Sigma) [219] and quantified with NIH Image-J software. The infarct volume (mm³) was determined as a percentage volume of the whole brain according to the formula: Infarct volume (%) = $100 \times [(V_c - V_i) / 2V_c]$; where V_c is the total volume of the contralateral hemisphere, and V_i is the noninfarcted volume of the ipsilateral hemisphere.

Western blotting

Each mouse brain was divided into two parts: contralateral (non-ischemic) hemisphere and ipsilateral (ischemic) hemisphere. The cortical gray matter was isolated and homogenized with RIPA lysis buffer (Santa Cruz, CA). The samples were then centrifuged at $15,000 \times g$ for 15 min at 4°C. The supernatants were collected and protein concentrations were determined using BCA protein assay kit (Pierce, Rockford, IL). Samples were separated on 4–15% Tris-HCl Ready SDS-polyacrylamide gels (Bio-Rad Laboratories, Hercules, CA), transferred onto PVDF membrane (Bio-Rad Laboratories), and incubated with the respective antibodies. Anti-ZO-1 (1:1000 dilution), anti-ZO-2 (1:500 dilution), anti-occludin (1:1000 dilution), anti-JAM-A (1:500 dilution), anti-claudin-1 (1:500 dilution), and anti-claudin-5 (1:500 dilution) antibodies were purchased from Zymed (San Francisco, CA). Anti-actin antibody (1:2500 dilution) was purchased from Sigma, and all secondary antibodies were from Santa Cruz Biotechnology. For visualization of detected proteins, immunoblots were analyzed using an ECL Western blot detection kit (Amersham Biosciences, Piscataway, NJ). Quantification of immunoreactive bands was performed by scanning densitometry using UN-SCAN-IT gel image analysis software (Silk Scientific, Orem, UT).

Real-Time RT-PCR

Real-time reverse transcriptase–polymerase chain reaction (RT-PCR) was performed as described previously [220]. Briefly, cortical grey matter samples were weighed and homogenized in 1 ml of TRIZOL reagent (Invitrogen, Carlsbad, CA) per 50 mg of tissue. Total RNA was extracted according to the manufacturer's protocol with an additional chloroform extraction and phase separation as well as an additional wash of the isolated RNA in 70% ethanol. Then, 1 µg of RNA was reverse-transcribed using the Reverse Transcription System (Promega, Madison, WI) in a total volume of 20 µL with random hexamer primers. The following conditions were employed for reverse transcription: 25°C for 10 min, 48°C for 30 min, and 95°C for 5 min. PCR amplification was performed using 3 µL of RT product, Taqman Universal PCR Master Mix (Applied

Biosystems, Foster City, CA), and predeveloped primer pairs and Taqman probes (Applied Biosystems) in a total volume of 25 μ L. The following thermocycling conditions were employed: 95°C for 10 min, followed by 95°C for 15 sec, and 60°C for 60 sec (for up to 40 cycles). Expression of mRNA was calculated and analyzed by the comparative CT method as described [221]. PCR amplification of mouse actin mRNA (a housekeeping gene) was performed for each sample to normalize mRNA levels of the target genes.

Measurements of glutathione (GSH) and superoxide levels

GSH content was determined by monochlorobimane (MCB; Invitrogen) staining. MCB conjugates with GSH through a reaction catalyzed by glutathione-S-transferase (GST) to form a fluorescent stable adduct (MCB-GSH) [222]. The levels of superoxide were detected by dihydroethidium (DHE; Invitrogen) staining. DHE is cell permeable, and at the presence of superoxide, it is converted to a fluorescent product ethidium bromide (EtBR), which then is trapped by intercalating with DNA [223]. Briefly, microvessels were incubated with 1 mL MCB solution (100 μ M) or 1 mL DHE solution (10 μ M) at 37°C for 90 min. Fluorescence was assessed using the Olympus BX61W1 confocal microscope (Olympus Corp., Tokyo, Japan).

Statistical analysis

Data are expressed as means \pm SEM. Statistical analysis was completed by using Sigma-Stat 2.03 (SPSS, Chicago, IL, USA). Two-tailed Student's t-test or one-way ANOVA followed by Student-Newman-Keuls post hoc test was used to compare mean responses among the treatments. A statistical probability of $p < 0.05$ was considered significant.

4. Results

TERT deficiency increases neurological deficit and infarct volume following pMCAO

As indicated in Figure 2.1, TERT-deficient mice exhibited significantly higher neurologic deficits 24 h post inducing pMCAO as compared to mice with normal TERT expression. The most affected locomotor functions in TERT $-/-$ mice were the parameters listed in category III as listed in Table 2.1. The functional locomotor scores were correlated with infarct volume as determined by TTC staining 24 h post the onset of pMCAO. As shown in Figure 2.2, the total infarct volume in the TERT $-/-$ mice was larger by an approximately 20% as compared to control mice.

Table 2.1. Locomotor scale to assess neurological deficit in mice

	Points	Category
Ipsilateral eyelid closed	1	I. Head and trunk
Hunched back	1	
Grasping (string) upon lifting the tail	1	II. Limb
Frontpaw withdrawal upon lifting the tail	1	
Hindpaw withdrawal upon lifting the tail	1	
Circling when walking	1	III. Whole-body activity
Body leaning to one side	1	
when placing on the table		
No spontaneous movement	1	
Total score	8	

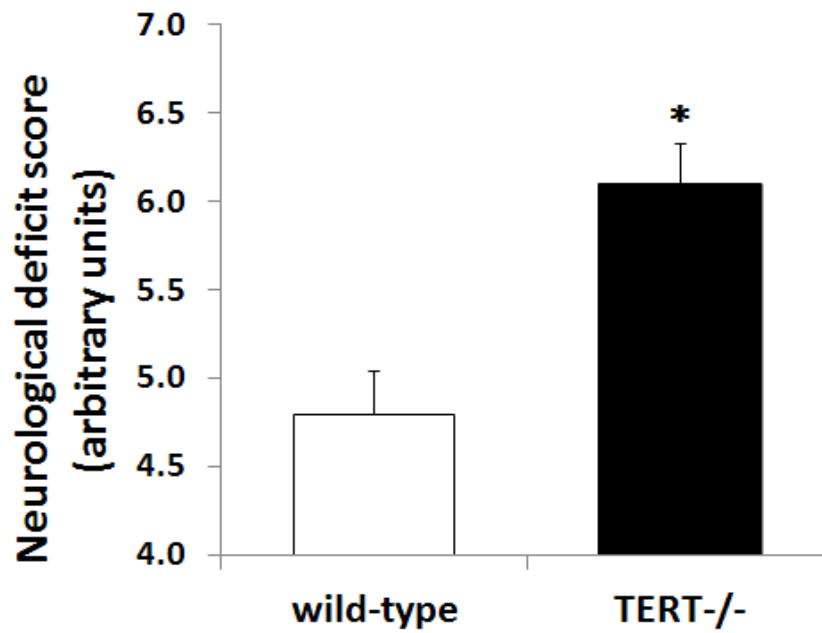


Figure 2.1. TERT deficiency potentiates neurological deficits induced by permanent occlusion of MCA. Neurological deficit was evaluated 24 hr post-pMCAO by using the eight-point score system listed in Table 2.1; n=10. *Significantly different compared with mice with normal TERT expression at $P < 0.05$.

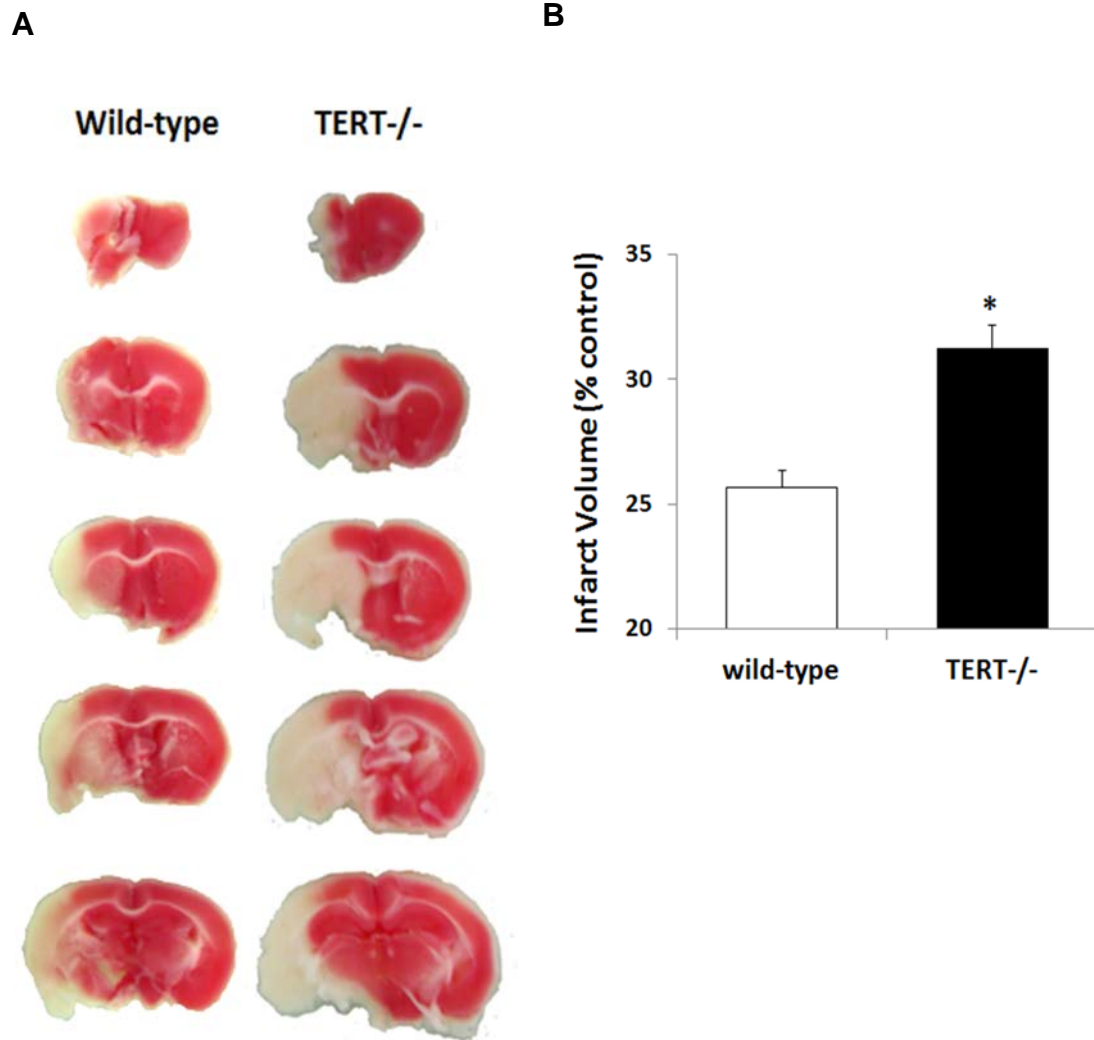


Figure 2.2. TERT deficiency increases infarct volume following pMCAO. Brain sections were stained with TTC 24 hr post-pMCAO. White areas correspond to damaged brain tissue resulting from infarct (A), and quantified results are depicted in the form of bar graphs (B); n=6. *Significantly different compared with mice with normal TERT expression at $P < 0.01$.

TERT deficiency enhances pMCAO-induced oxidative stress in brain microvessels

In order to address potential mechanisms of increased infarct volume in TERT $-/-$ mice, our studies focused on the involvement of the BBB and induction of oxidative stress in brain microvessels. Superoxide levels were detected in isolated brain microvessels 24 h post induction of pMCAO (Fig. 2.3). The most pronounced increase in superoxide levels was observed in ipsilateral hemispheres of TERT $-/-$ mice with induced pMCAO (~4.5 times of control levels). Although superoxide levels were also elevated in ipsilateral hemispheres of control mice with pMCAO, these changes were approximately 2-fold lower as compared to the corresponding values in TERT $-/-$ animals.

Induction of pMCAO resulted in decreased glutathione levels in ipsilateral hemispheres in both control and TERT $-/-$ mice. However, this effect was significantly enhanced in TERT-deficient animals, indicating severely diminished antioxidant protection in this group of mice. In contrast to control mice, glutathione levels were also diminished in contralateral hemispheres in TERT $-/-$ animals (Fig. 2.4).

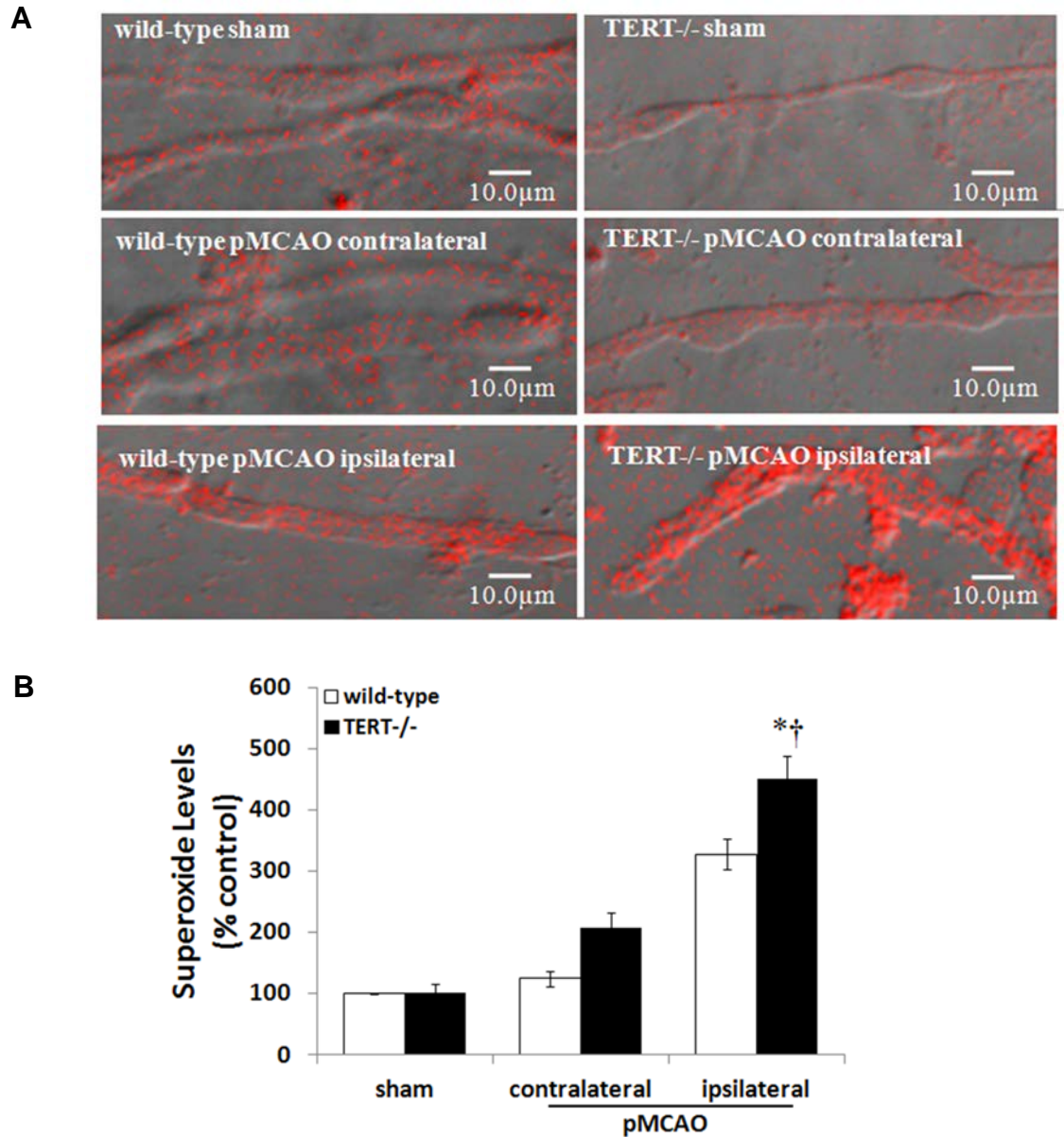


Figure 2.3. pMCAO-induced oxidative stress in brain microvessels is enhanced in TERT-deficient mice. Twenty-four hours after pMCAO or sham operation, brain microvessels were isolated and stained for superoxide levels with DHE (red). All images were acquired with a X60 oil-immersion lens under identical instrument settings. Merged micrographs of DHE staining and phase-contrast micrographs localized superoxide within the microvessels (A). The intensity of DHE fluorescence was quantified and depicted in the form of a bar graph (B). DHE fluorescence values in microvessels from ipsilateral and contralateral hemispheres of sham-operated mice were the same and the results were pooled; $n=6-8$. *Significantly different compared with sham operated mice at $P < 0.05$. †Results in the TERT-/- group are statistically different from those in the corresponding hemispheres of mice with normal TERT expression at $P < 0.05$.

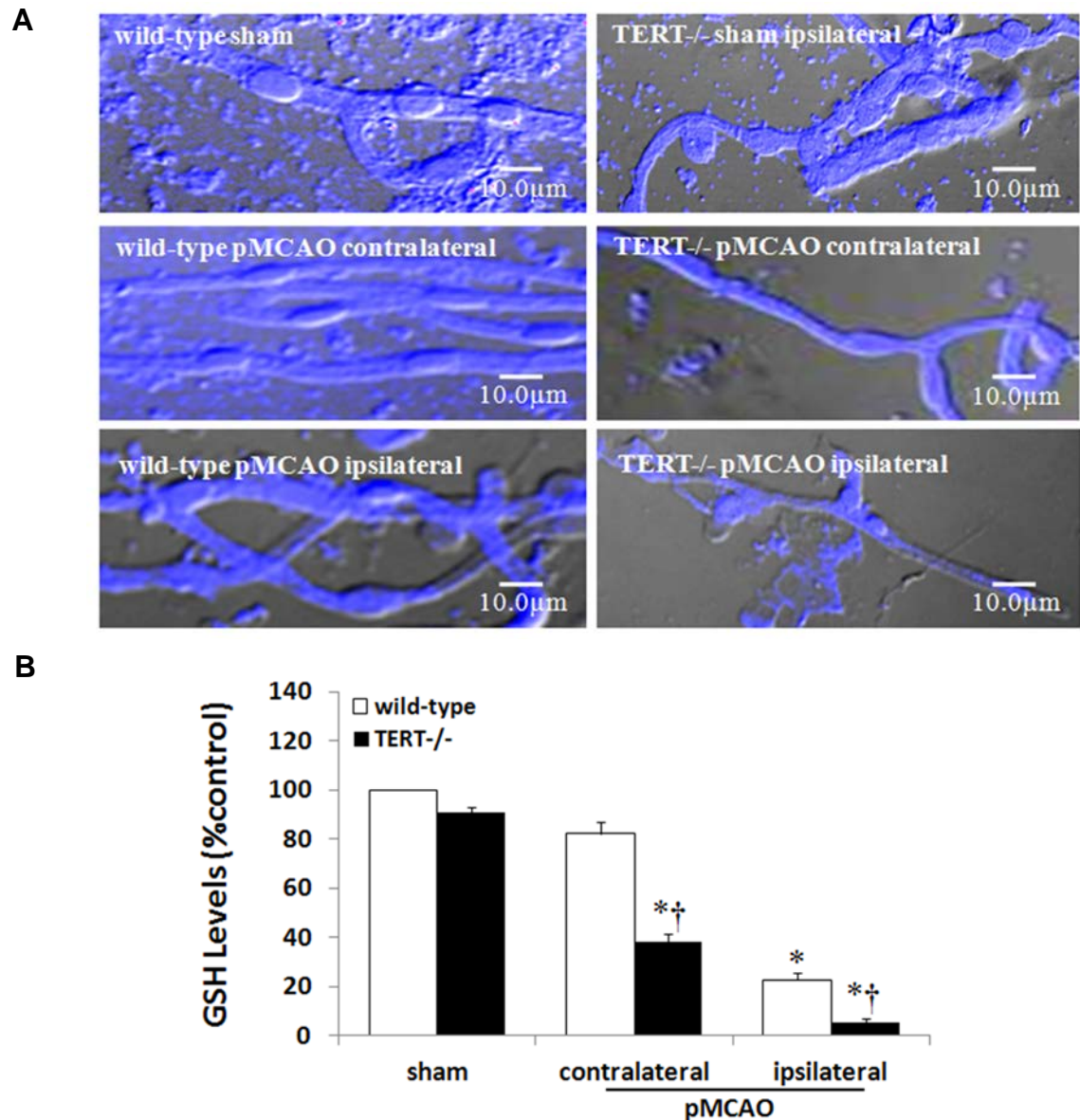


Figure 2.4. TERT deficiency potentiates pMCAO-induced glutathione depletion in brain microvessels. Twenty-four hours after pMCAO or sham operation, brain microvessels were isolated and stained for glutathione levels with MCB (blue). Images were acquired as for Figure 2.3. Merged micrographs of glutathione staining and phase-contrast micrographs localized glutathione within the microvessels (A). The intensity of glutathione staining was quantified and depicted in the form of a bar graph (B); $n=6-8$. Fluorescence intensities in microvessels from ipsilateral and contralateral hemispheres of sham-operated mice were the same, and the results were pooled. *Significantly different compared with sham operated mice at $P < 0.05$. †Results in the TERT-/- group are statistically different from those in the corresponding hemispheres of mice with normal TERT expression at $P < 0.05$.

TERT deficiency differentially influences pMCAO-induced changes in TJ protein expression

TJs are the main structural elements of cerebral microvessels that regulate the integrity of the BBB. Therefore, we evaluated the effects of TERT deficiency on the levels of TJ proteins in mice with pMCAO. As illustrated in Figure 2.5, occludin and ZO-1 exhibited different expression patterns following MCA occlusion. Occludin expression was significantly increased in the ipsilateral hemisphere of control mice with pMCAO; however, the level of this TJ protein was not affected in the brains of TERT $-/-$ mice with pMCAO as compared to sham controls. In contrast, expression of ZO-1 decreased to almost negligible levels in the ipsilateral hemisphere of TERT deficient mice with pMCAO. Similar tendency was observed in mice with normal TERT expression; however, these changes were significantly less pronounced. Expression of other TJ proteins, such as ZO-2, JAM-A, claudin-5, and claudin-1, was not affected 24 h post pMCAO in either TERT $-/-$ or control mice (data not shown).

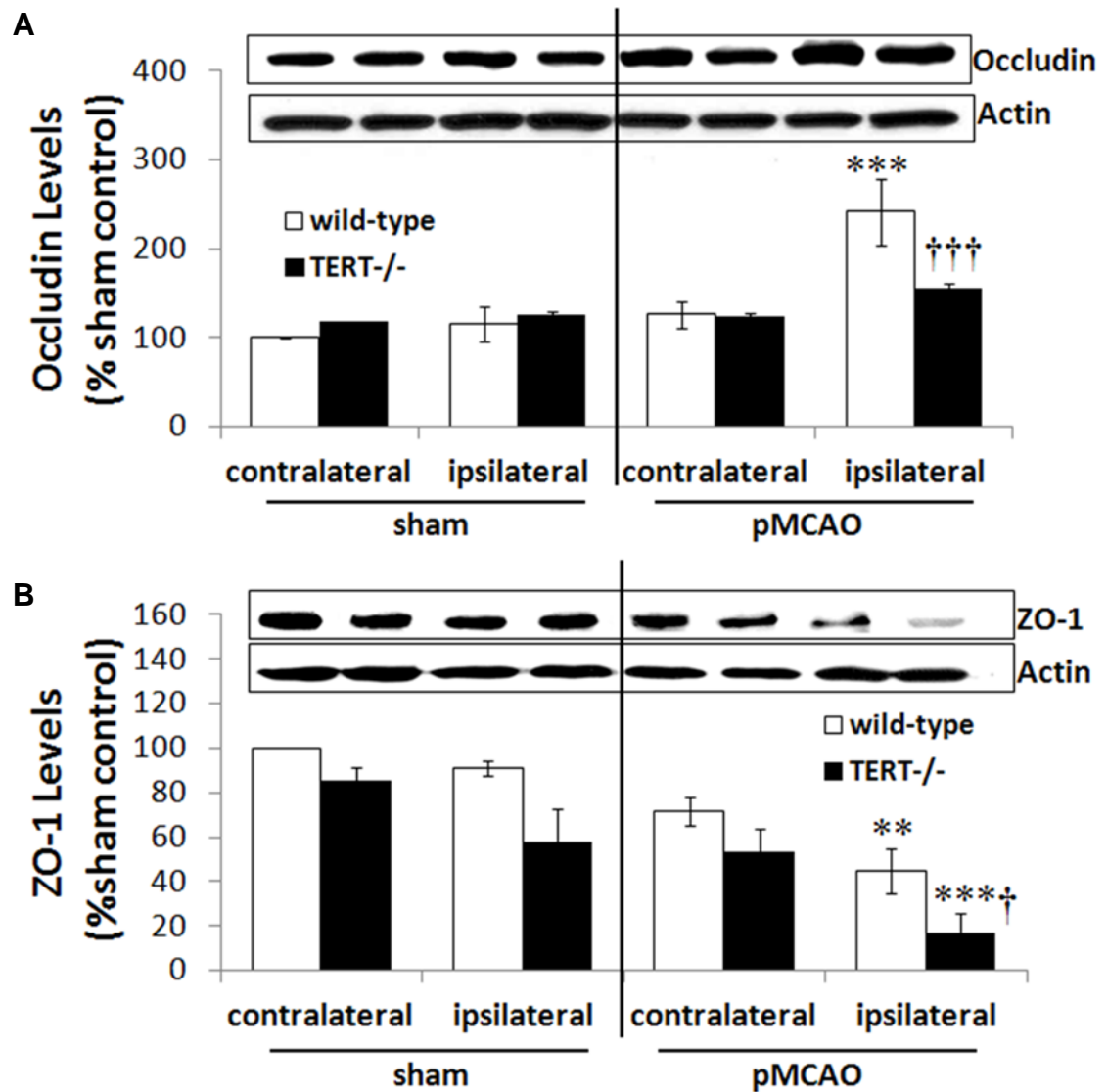


Figure 2.5. TERT deficiency differentially influences pMCAO-induced changes in TJ protein expression. Expression of TJ proteins occluding (A) and ZO-1 (B) was analyzed by Western blotting 24 hr after pMCAO or sham operation separately in ipsilateral (ischemic) and contralateral (nonischemic) hemispheres. The blots are representative images from four mice per group, and the quantified results are depicted in the form of bar graphs; n=4. Significantly different compared with the respective hemispheres of sham operated mice at **P < 0.01, ***P < 0.001. Results in the TERT-/- group are statistically different from those in the corresponding hemispheres of mice with normal TERT expression at †P < 0.05, †††P < 0.001.

TERT deficiency potentiates inflammatory responses following pMCAO

We also examined brain tissues for mRNA expression of TNF- α , IL-1 β , and ICAM-1 24 h post induction of pMCAO. The choice of these inflammatory mediators includes cytokines (TNF- α and IL-1 β), and an adhesion molecule (ICAM-1). These agents are critical in the induction of inflammatory reactions in the vascular endothelium by stimulating leukocyte recruitment, adhesion, and transendothelial migration. pMCAO resulted in a significant elevation of mRNA levels of all studied inflammatory mediators in ipsilateral hemispheres of TERT $-/-$ and control mice (Fig. 2.6A–C). Importantly, these levels were more advanced in TERT deficient mice. Specifically, mRNA levels of TNF- α , IL-1 β and ICAM-1 in the ischemic hemisphere of TERT $-/-$ mice increased 3, 2.6, and 2.4-fold, respectively, as compared to the corresponding hemisphere of control mice with pMCAO. TNF- α and ICAM-1 mRNA expression was also elevated in contralateral hemispheres of TERT $-/-$ mice.

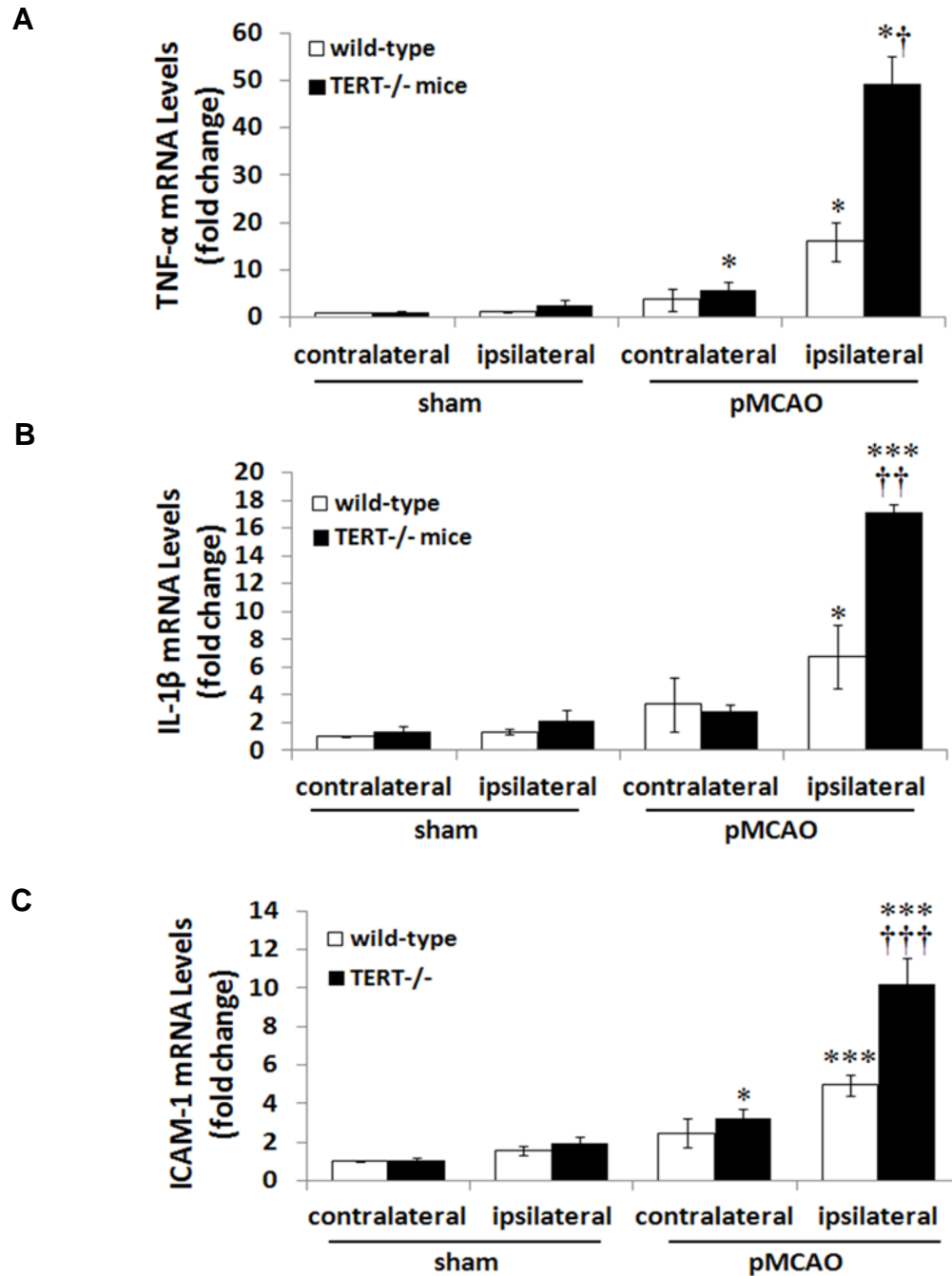


Figure 2.6. TERT deficiency potentiates pMCAO-induced inflammatory responses. Experiments were performed as described for Figure 2.5. mRNA levels of TNF- α (A), IL-1 β (B), and ICAM-1 (C) were determined by real-time PCR; n=4. Significantly different compared with the corresponding hemispheres of sham operated animals at *P < 0.05, ***P < 0.001. Results in the TERT-/- group are statistically different from those in the corresponding hemispheres of mice with normal TERT expression at †P < 0.05, ††P < 0.01, †††P < 0.001.

5. Discussion

The BBB is a dynamic interface between the peripheral circulation and the CNS. In the present study, we provide *in vivo* evidence for the role of TERT deficiency in the BBB dysfunction and brain injury during ischemic stroke. Specifically, TERT $-/-$ mice showed severe neurological deficits and increased infarct volume as compared to animals with normal TERT expression 24 h post induction of pMCAO. These alterations were closely associated with increased oxidative stress in brain capillaries, alterations of TJ protein expression and increased inflammatory responses.

The presence of telomerase activity in the brain during development characterizes the role of this enzyme in neuronal differentiation and survival [224]. Both telomerase and TERT levels are generally decreased during embryonic and early postnatal development in somatic cells. Particularly, telomerase activity is reduced as proliferation of neuroblasts is decreased, whereas TERT levels are reduced in conjunction with neuronal differentiation and natural cell death [83]. This dissociation between telomerase activity and TERT levels suggests that TERT may function to promote cell survival through a mechanism other than maintaining telomere length [225]. In addition, recent reports from epidemiological and genetic studies indicate that humans with shorter telomeres than average are at higher risk of heart disease, stroke and degenerative diseases [77].

Our study indicated that TERT $-/-$ mice are more susceptible to ischemic brain injury. To support these observations, it was recently reported that TERT transgenic mice were characterized by significantly reduced infarct volume after MCAO and protective effects against NMDA-induced neurotoxicity [86]. In addition, TERT transgenic mice exhibited a reduced myocardial infarct area after coronary artery ligation, and exogenous TERT expression in cardiac myocytes promoted survival by protection against apoptosis [212].

Impairment of TJs and the accompanied dysfunction of the BBB are typical events during cerebral ischemia [226]. Indeed, our studies indicated a marked loss of ZO-1 in the ipsilateral hemisphere of the mice with pMCAO. Importantly, this effect was

potentiated in TERT $-/-$ mice as compared to control animals. ZO-1 is essential for TJ assembly [227] as it forms a scaffold complex with other cytosolic accessory proteins, such as ZO-2 and AF6, to anchor integral membrane proteins to the actin cytoskeleton. Disruption of ZO-1 at TJs has been observed in response to cytokines [228] and hypoxia [229], and has been correlated with increased transendothelial permeability. In the context of stroke, an ex vivo study of cerebral ischemia identified translocation and decreased expression of occludin and ZO-1 in brain capillaries after microsphere-induced cerebral embolism in rats [230]. In contrast, we observed increased occludin levels in control mice following pMCAO but no apparent changes in TERT $-/-$ mice. The influence of alterations of occludin expression on the BBB integrity is not fully understood. For example, increased occludin levels frequently correspond with decreased permeability across the BBB [231]; however, others reported also the opposite phenomenon [232]. It appears that interactions of occludin with other TJ proteins, including ZO-1, may have more prominent consequences on the BBB integrity than changes in levels of this TJ protein.

The loss of blood supply to the brain associated with the onset of pMCAO results in a cascade of events including inflammation and induction of oxidative stress around the infarcted region. Inflammatory responses include upregulation of cytokines (e.g. TNF- α and IL-1 β) [233] which have been shown to precede BBB permeability and promote leukocyte infiltration into the brain tissue through induction of adhesion molecules on the surface of endothelial cells [234]. In addition, activated microglia can release a variety of inflammatory mediators upon brain ischemia, while blood-borne leukocytes can migrate into the brain and subsequently contribute to production of inflammatory substances, increased permeability of the BBB and secondary injury to the brain tissue [41, 235]. Our novel findings illustrate that pMCAO resulted in more pronounced expression of TNF- α , IL-1 β , and ICAM-1 in TERT deficient mice as compared to mice with normal TERT expression. These results are in agreement with the report that TNF- α -mediated increase in monocyte adhesion in senescent human endothelial cells can be attenuated in TERT transfected cells [236], indicating a relation between telomerase activity and endothelial dysfunction.

Another novel observation of the present study indicates that superoxide production and decreased GSH levels in brain capillaries are more pronounced in TERT $-/-$ mice as compared to controls with normal TERT expression upon induction of pMCAO. Increased oxidative stress is known to stimulate redox-regulated signaling pathways that dysregulate TJ protein expression and affect the BBB integrity [237]. Persistent oxidative stress can lead to diminished antioxidant protection [238, 239], as observed in the present study by decreased GSH levels in brain microvessels. GSH is a critical antioxidant in vascular endothelial cells and its depletion is strongly associated with BBB dysfunction [240]. In agreement with our study, recent evidence emphasizes the role of telomerase in the protection against induction of oxidative stress. For example, overexpression of TERT increased oxidative stress resistance, improved antioxidant defense, and differentiation capacity in mouse embryonic stem cells [241]. Overexpression of TERT also resulted in improved mitochondrial functions, protecting against production of superoxide radicals and increased cellular ROS levels [92]. To further demonstrate the interrelationship between oxidative stress and telomerase activity, it was demonstrated that cellular oxidation can impair telomerase activity and accelerate the telomere shortening in endothelial [242, 243] and vascular smooth muscle cells [244], as well as result in telomerase exclusion from the nucleus to the mitochondria [245, 246].

Increased oxidative stress and diminished antioxidative protection in brain microvessels of TERT $-/-$ animals may further contribute to the disruption of the BBB integrity as observed in the present study. In fact, it was demonstrated that oxidative stress can alter TJs at the BBB level by alterations of phosphorylation and expression of TJ proteins [232, 247-249]. Increased levels of ROS can also stimulate NF- κ B activation, leading to increased production of inflammatory cytokines and adhesion molecules [250], such as TNF- α , IL-1 β , and ICAM-1 evaluated in the present manuscript. In the present study, elevated superoxide levels were mostly limited to the ipsilateral hemisphere following the pMCAO-based experimental stroke model. In agreement with these results, increased expression of inflammatory genes and disruption of TJ proteins were also mostly affected in ipsilateral hemisphere.

In conclusion, the results of the present study indicate that TERT deficiency potentiates the BBB dysfunction, inflammatory responses, and oxidative stress associated with experimental stroke induced by the permanent occlusion of MCA. Importantly, these alterations were associated with increased neurological deficit and infarct volume. The present results emphasize the importance of TERT in the protection of brain injury against ischemic stroke.

Chapter three: Cerebrovascular toxicity of PCB153 is enhanced by binding to silica nanoparticles

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1. Synopsis

Environmental polychlorinated biphenyls (PCBs) are frequently bound onto nanoparticles (NPs). However, the toxicity and health effects of PCBs assembled onto nanoparticles are unknown. The aim of this study was to study the hypothesis that binding PCBs to silica NPs potentiates PCB-induced cerebrovascular toxicity and brain damage in an experimental stroke model. Mice (C57BL/6, males, 12-week-old) were exposed to PCB153 bound to NPs (PCB153-NPs), PCB153, or vehicle. PCB153 was administered in the amount of 5 ng/g body weight. A group of treated animals was subjected to a 40 min ischemia, followed by a 24 h reperfusion. The blood–brain barrier (BBB) permeability, brain infarct volume, expression of tight junction (TJ) proteins, and inflammatory mediators were assessed. As compared to controls, a 24 h exposure to PCB153-NPs injected into cerebral vasculature resulted in significant elevation of the BBB permeability, disruption of TJ protein expression, increased proinflammatory responses, and enhanced monocyte transmigration in mouse brain capillaries. Importantly, exposure to PCB153-NPs increased stroke volume and potentiated brain damage in mice subjected to ischemia/reperfusion. A long-term (30 days) oral exposure to PCB153-NPs resulted in a higher PCB153 content in the abdominal adipose tissue and amplified adhesion of leukocytes to the brain endothelium as compared to treatment with PCB153 alone. This study provides the first evidence that binding to NPs increases cerebrovascular toxicity of environmental toxicants, such as PCB153.

2. Introduction

Polychlorinated biphenyls (PCBs) are organochlorinated chemicals that were mass produced for industrial usage in the U.S. for approximately 50 years [251]. Environmental exposure to PCBs is ongoing as a result of continued use and disposal of products containing these toxicants [252], as well as prevalent bioaccumulation of PCBs in the biosphere and in the food chain [253]. PCBs present in the environment can bind to organic particles in water, sediments, soil, and atmospheric particulates. In contaminated areas in New Bedford Harbor (MA), average PCB concentration was determined to be 2.3 mg/L in water and 351 µg/kg in sediments [254]. The average PCB levels in the air and soil samples collected in Anniston (AL) were 62.8 ng/m³ and 2.8 mg/kg, respectively [255]. Importantly, elevated environmental levels of PCBs were associated with increased levels in serum in the exposed population and higher prevalence to disease development [132, 256]. Evidence suggests that exposure to PCBs may increase the incidence of stroke [14] and worsen stroke outcome [257]. Importantly, highly chlorinated ortho-PCBs preferentially accumulate in the brain and thus induce prominent neurotoxic effects [258]. The most representative compound from this group is 2,2',4,4',5,5'-hexachlorobiphenyl (PCB153), which is common in environmental samples and accounts for 15–30 % of total PCB content in most human samples [259].

When released from environmental sources, PCBs can be adsorbed onto suspended particles present in the air, such as ultrafine (nano-size particles) or fine particles. It has been reported that PCBs can bind to indoor and outdoor dust with the particle size ranging from 0.95 µm to 1.5 µm [260-262]. Nanoparticles (NPs) and fine particles are able to penetrate the alveolar-capillary barrier of the lung to access circulating blood cells (e.g. erythrocytes) [263]. Results from animal experiments indicated that ultrafine particles can translocate into the systemic circulation [264] and the brain [149] through inhalation or nasal instillation. Thus, particulate components from the ambient air may act as effective carriers for various environmental pollutants entering the brain.

The blood–brain barrier (BBB) is a chemical and physical barrier composed of brain endothelial cells, which are connected by tight junctions (TJs), and interact with

other cells and basement membrane of the neurovascular units [265, 266]. The presence of intact TJs is essential in maintaining a functional BBB [265]. Disruption of the BBB is associated with several acute and chronic disorders of the central nervous system (CNS), including stroke, ischemia/reperfusion, hypoxia/reoxygenation and cerebrovascular dysfunction [52, 266, 267]. In addition, age and conditions linked to aging, including hypertension and brain ischemia, can contribute to alterations of BBB functions. The BBB contains the specialized carriers for glucose, amino acids, purine bases, nucleosides and other substances, which ensure adequate delivery of nutrients, hormones, and neurotransmitters. At the same time, the brain endothelium acts as an effective barrier to prevent the circulating toxic agents entering the brain parenchyma. Thus, age-related BBB dysfunction may impair the transport of nutrients and metabolites to the brain, whereas the leaky barrier allows the circulating toxicants access the brain tissue. Moreover, genetic variances may affect the BBB functions and contribute to the disease etiology. For instance, heterozygous mutation in the *glut1* gene results in glucose transporter (GLUT-1) deficiency syndrome [268], which impairs the transport of glucose across the BBB. The cells composing the BBB can also interact with NPs. It was demonstrated that NPs have ability to cross the BBB and accumulate in brain parenchyma [169].

With the robust development of nanotechnology, nanotoxicity is also gaining increased attention. Surface modifications of NPs markedly affect their biological and toxicological properties. For example, neutral and anionic NPs at low concentrations appear to have no effect on BBB integrity, whereas high concentrations of positively or negatively-charged NPs disrupted the BBB. Anionic NPs were preferentially taken up by the brains as compared to neutral or positively-charged NPs [269]. Other unique functionalities of NPs, including high surface area, make NPs a target for other environmental contaminants, such as PCBs. Nevertheless, there are no research reports on cerebrovascular toxicity of PCBs bound to NPs. In the present study, we demonstrate for the first time that such binding results in increased cerebrovascular toxicity of PCB153, leading to enhanced brain injury in experimental stroke model.

3. Materials and methods

Characterization of silica NPs and binding PCB153 to NPs

Silica NPs were purchased from NanoAmor (Houston, TX). In order to determine the effect of different media on particle size distribution, particles were dispersed in distilled water, phosphate buffered saline (PBS), or EBM-2. Then, the diameter size and the polydispersity index (PDI) were assessed by dynamic light scattering (DLS) using a Malvern Instruments Zetasizer Nano ZS.

In order to assemble PCB153 onto NPs, silica NPs (80 mg) were mixed with 10 mg of PCB153 (2,2',4,4',5,5'- hexachlorobiphenyl, AccuStandard, New Haven, CT) in acetone and sonicated. Acetone was allowed to evaporate and the mixture of NPs with PCB153 was resuspended in water PBS or EBM-2. Sonication was applied during the process to minimize the particles' aggregation. The suspension was centrifuged at 12,000 rpm for 5 min. The supernatant was collected to analyze the final concentration of PCB153 by gas chromatography/mass spectrometry (GC/MS). Control NPs were prepared using the same procedure, without adding PCB153.

Animals and experiment groups

All experiments were performed following the protocol approved by the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Mice (C57BL/6, 12-week-old, male) were anesthetized with isoflurane and then infused with: a) PCB153 bound to silica NPs (PCB153-NPs), b) PCB153 dissolved in 0.01 % DMSO (PCB153), c) silica NPs alone, or d) vehicle (DMSO or PBS). Sham controls were subjected to surgical procedures without additional treatment. All injections were performed through the internal carotid artery (ICA) to ensure drug delivery into the brain. PCB153 was administered in the amount of 5 ng/g body weight. This design simplified the routes of exposure and potential surface modification of NPs, which may occur when passing the gastrointestinal track, skin, or airway passages. Direct injections into the bloodstream

allowed for precise dosing to evaluate whether binding of PCB153 to NPs influences their toxicological properties.

In selected experiments, mice were exposed to PCB153-NPs or PCB153 alone by oral gavage at the dose of 5 ng/g once a day for 30 days. Such treatment mimics chronic exposure to PCBs through food chain. Adipose tissue was then collected and analyzed for PCB153 levels.

Surgical procedures

Injections via the internal carotid artery (ICA) and the installation of the vessel port were performed as described earlier by our group [270]. The focal ischemia stroke model was based on a 40 min occlusion of the middle cerebral artery (MCA), confirmed by a sharp decline in cerebral blood flow, followed by 24 h reperfusion. Then, the brains were removed, sectioned into 1 mm slides, and the infarct area was visualized by 2,3,5-triphenyltetrazolium chloride (TTC) staining. The infarct volume was quantified by ImageJ analysis software as described earlier [271].

Cranial windows were installed to observe leukocyte interactions with cerebral vessels. Under anesthesia, the left parietal bone was exposed by a midline skin incision, followed by a craniotomy (2.5 mm diameter) 2.5 mm posterior from the bregma and 2.5 mm lateral from the midline. The dura mater was removed to expose the underlying pial vasculature. A glass coverslip was placed and sealed with dental cement over the exposed brain tissue, which was suffused with artificial cerebrospinal fluid. Attachment of leukocyte to the brain endothelium was assessed as described earlier [272] using rhodamine 6G (Rho6G) chloride to label circulating leukocytes.

Monocyte transmigration assay in vivo

Murine monocyte/macrophage cells J774.1 were cultured in DMEM medium (Invitrogen, Carlsbad, CA) supplemented with 10 % FBS and antibiotics (penicillin, 100

U/ml; and streptomycin, 100 µg/ml, Invitrogen). Monocytes were labeled with 20 µM CFDA-SE (Vybrant CFDA-SE Cell Tracer Kit, Invitrogen), a dye which passively diffuses into cells and yields fluorescence when its acetate groups are cleaved by intracellular esterases. Mice were injected into the ICA with labeled monocytes at a concentration of 0.5×10^6 cells, which were then allowed to circulate for 24 h. Brains were sliced (20 µm thick) and stained to visualize the interaction of labeled monocytes with the brain endothelium. Staining for claudin-5 was employed to visualize the brain capillaries. Images were acquired using a Nikon fluorescence microscope (Nikon, Melville, NY) equipped with a SPOT RT camera and software (Diagnostic Instruments, Sterling Heights, MI).

Analysis of PCB153 levels in adipose tissue

Tissue levels of PCB153 were analyzed as described previously [135]. Briefly, adipose tissue samples (0.2–0.5 g) were spiked with surrogate standards (PCB65 and PCB166) and mixed with 1.0 g diatomaceous earth. The mixtures were then homogenized and extracted with ASE 200 accelerated solvent extractor (Dionex Corporation, Sunnyvale, CA). After extraction with hexane, the extracts were concentrated using Rotavapor, vortexed thoroughly, left overnight to achieve phase separation, and the hexane layer containing PCB153 was collected. The extracted aliquots were further concentrated and 10 µL of 1.0 ng/µL of internal standard PCB209 was added to each sample and transferred to a glass microvial for analysis. Gas chromatographic analysis was performed with a GC-µECD system (Agilent GC 6890 N, autosampler G2913A, µECD detector) (Agilent Technologies, Santa Clara, CA). PCB153 was identified on the basis of the retention time relative to standards. Quantification was achieved based on calibration curves obtained using PCB standards, the recovery efficiency calculated from the surrogates, and the sample weight.

BBB permeability assay

Animals were injected i.p. with 200 μ L 10 % sodium fluorescein in PBS. Fifteen minutes later, blood was collected via cardiac puncture. Animals were transcardially perfused with 0.9 % saline, the brains were harvested, and homogenized in PBS (1/10; w/v). Fluorescence was determined using excitation at 485 nm and emission at 530 nm. The permeability results were presented as a ratio of brain to plasma fluorescence intensity.

Immunoblotting and immunoreactivity

Brain microvessels were isolated as described previously [144] and either lysed for immunoblotting or smeared on the glass slides for immunoreactivity assessments. Lysed microvessels (50 μ g protein per sample) were electrophoresed on 4–15 % Tris–HCl Ready SDS-polyacrylamide gel (Bio-Rad Laboratories, Hercules, CA), transferred onto PVDF membranes (Bio-Rad Laboratories), and incubated with specific primary antibodies. Anti-occludin and anti-claudin-5 antibodies were purchased from Invitrogen, anti-actin antibody was from Sigma, and all secondary antibodies were from Santa Cruz Biotechnology.

Brain microvessels smeared on the glass slides were incubated with anti-occludin and anti-claudin-5 antibodies for tight junction (TJ) immunoreactivity studies.

Real-time RT-PCR

Freshly isolated microvessels were resuspended in 200 μ L of TRIZOL reagent (Invitrogen) and total RNA was extracted according to the manufacturer's instructions. Then, 1 μ g of RNA was reverse-transcribed using the Reverse Transcription System (Promega, Madison, WI). PCR amplification was performed using 3 μ L of RT product, Taqman Universal PCR Master Mix (Applied Biosystems, Foster City, CA), and the pre-developed primer pairs and Taqman probes (Applied Biosystems) in a total volume of 25 μ L. Expression of mRNA was calculated and analyzed by the comparative CT method as described earlier [221].

Cultures of human brain microvascular endothelial cells and in vitro transendothelial migration

Human brain microvascular endothelial cells (hCMEC/D3 cell line) were cultured as described previously [273]. For transendothelial migration, 1×10^5 hCMEC/D3 cells were cultured on a Transwell filter until reaching confluency. Treatment factors were the same as in animal experiments; however, final concentration of PCB153 was 1.6 μ M. Human monocytic THP-1 cells were stained with calcein acetoxymethyl (calcein AM) (5 μ M) for 15 min and added in the amount of 2.5×10^5 on top of hCMEC/D3 monolayers for 4 h at 37 °C. Fluorescence originating from the migrating THP-1 cells was measured in 200 μ L aliquots collected in the lower compartment of the Transwell system at 485 nm excitation and 530 nm emission.

Statistical analysis

Statistical analysis was completed by using Sigma-Stat 2.03 (SPSS, Chicago, IL). One-way ANOVA, followed by Student- Newman-Keuls post hoc test or two-tailed Student's t-test, was used to compare mean responses among the treatments. A statistical probability of $p < 0.05$ was considered significant.

4. Results

Characterization of NPs and PCB153-NPs

Nanoparticles are known to agglomerate in aqueous solutions. Both NPs and PCB153-NPs agglomerated in water PBS and EBM-2 generating aggregates with the average hydrodynamic diameter of ~250 nm for PCB153-NPs and ~350 nm for silica NPs alone (Table 3.1). Data reported here are based on Cumulants analysis. Z-average diameter is the mean diameter based on the intensity of scattered light, and the PDI describes the width of the particle size distribution, calculated as $PDI = \sigma^2 / Z_D^2$, where σ is

the standard deviation and Z_D is the Z average mean size. An increase in the Z-average diameter is an indication of particle aggregation.

As shown in Table 3.1, the size and PDI of NPs and PCB153-NPs vary in different media. Particles dispersed in PBS and EBM-2 displayed higher PDI value than in water, indicating less uniform dispersion. This may attribute to the ionic strength (IS), pH, or interaction of particles with serum proteins in cell culture medium. Therefore, during material preparation NPs were first dispersed in water and then diluted to final concentration in PBS.

Table 3.1. Hydrodynamic particle diameters of NPs in different media as determined by dynamic light scattering

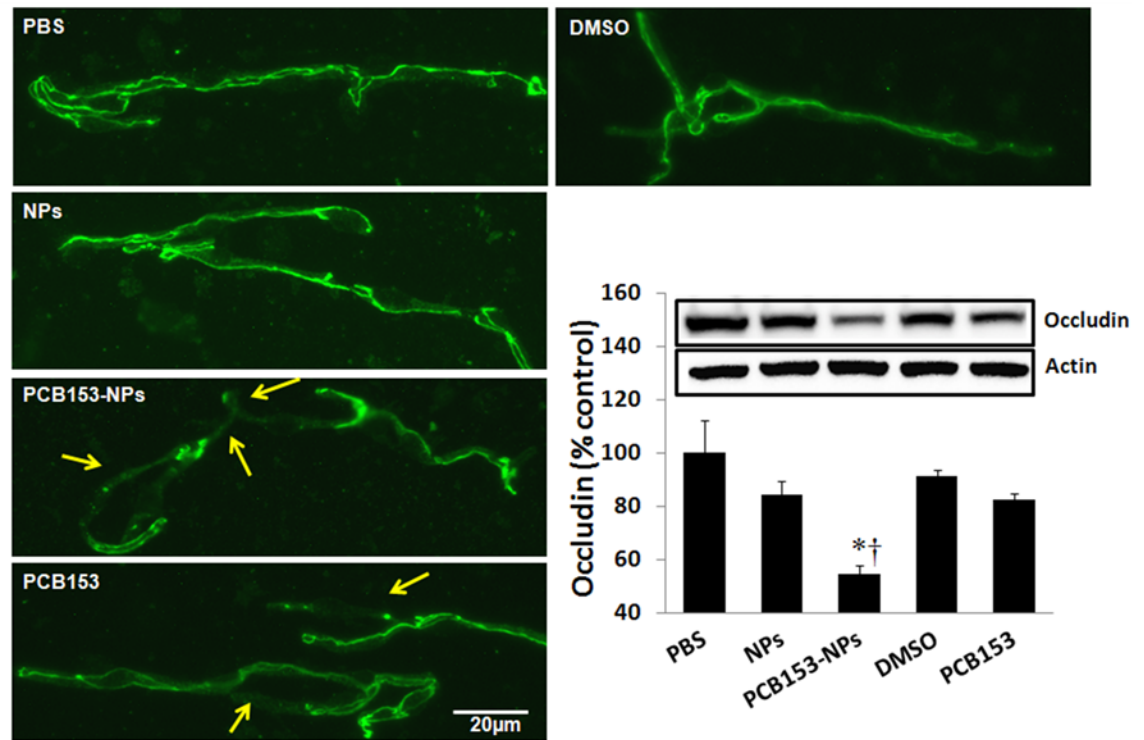
Particles	Z-Average Diameter (nm)	PDI
NPs		
Water	346.5	0.246
PBS	346.3	0.610
EBM-2	333.6	0.294
PCB153-NPs		
Water	246.7	0.277
PBS	265.8	0.442
EBM-2	239.5	0.511

Disruption of TJ protein expression and the BBB integrity is enhanced in mice exposed to PCB153-NPs

TJs are critical elements that regulate the integrity of the BBB. There was a tendency towards diminished levels of occludin after exposure to NPs or PCB153 alone; however, quantified results indicated that these changes did not reach statistical significance. Importantly, administration of PCB153-NPs resulted in a significant decrease in expression of occludin and claudin-5 in brain microvessels (Fig. 3.1 A and B, respectively) as determined by immunoblotting and immunoreactivity.

Alterations of TJ molecular properties have been associated with disruption of BBB integrity [266]. Therefore, we evaluated the influence of PCB153 and/or NPs on the BBB function using an assay based on permeability of a fluorescent marker sodium fluorescein. As indicated in Fig. 3.2, exposure to PCB153-NPs significantly increased the BBB permeability. Such effects were not observed in animals treated with equimolar amount of PCB153 or NPs alone.

A



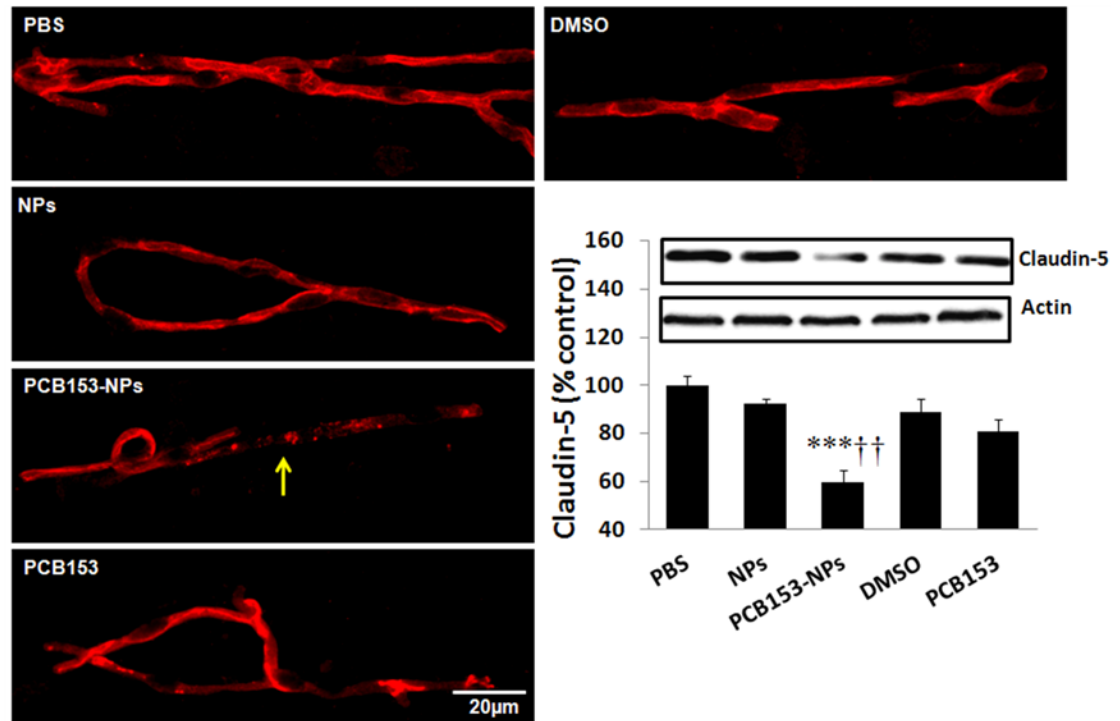
B

Figure 3.1. Treatment with PCB153-NPs disrupts expression of tight junction proteins and BBB integrity. Mice were exposed to PCB153-NPs by infusion into the internal carotid artery (ICA) at the dose of 5 ng PCB153/g body weight bound to 1.04×10^5 silica NPs. Control mice were infused with the same amounts of NPs, PCB153 dissolved in DMSO, or vehicle (PBS or 0.01 % DMSO). Brain microvessels were isolated 24 h post treatment and analyzed for occludin (A) and claudin-5 (B) expression by immunoblotting and immunofluorescence. Immunoreactivity of occluding and claudin-5 was stained in green and red, respectively; scale bar =20 μm. The blots in A and B are representative images from all experiments and the quantified results are depicted in the form of bar graphs. Arrows indicate disrupted continuity of tight junction proteins. Results are mean±SEM, n=4. *Significantly different as compared to control groups at * $p < 0.05$ or *** $p < 0.001$. †Results in the PCB153-NP group are statistically different from those in the PCB153 group at † $p < 0.05$ or ††† $p < 0.001$.

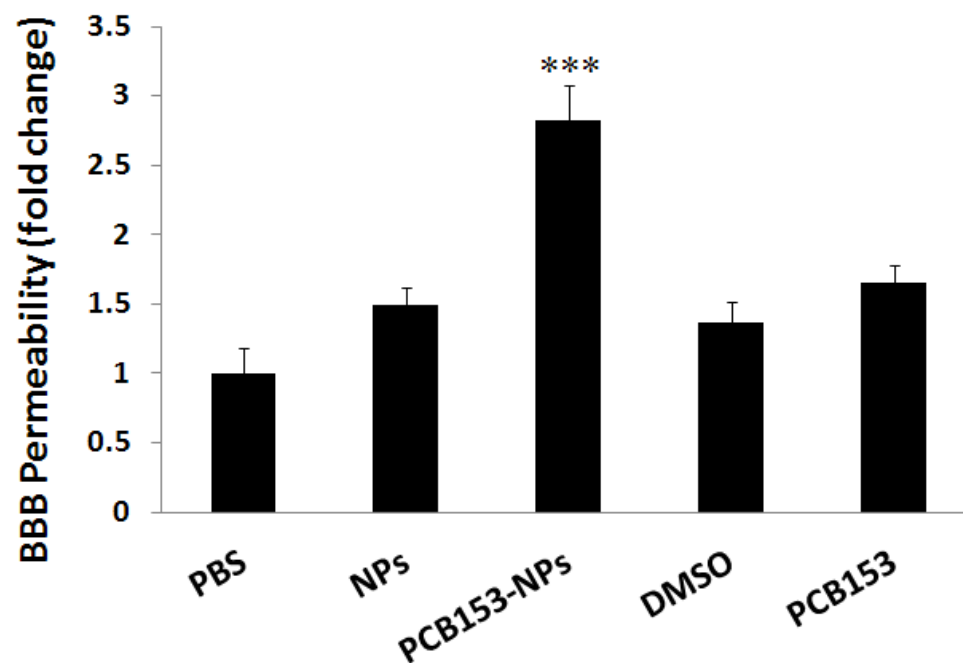


Figure 3.2. Treatment with PCB153-NPs increases permeability of the BBB. Mice were injected with vehicle, PCB153 and/or NPs as in Figure 3.1. BBB permeability was evaluated using sodium fluorescein 24 h post treatment. Results are mean \pm SEM, n=4. ***Significantly different as compared to other groups at $p<0.001$.

Exposure to PCB153-NPs potentiates inflammatory responses in brain capillaries

Stimulation of proinflammatory responses is both the prominent feature of PCB-induced toxicity in the brain [135] and an important element of stroke pathology. Therefore, we determined the effects of PCB153 and/or NPs on mRNA expression of proinflammatory cytokines tumor necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β) as well as adhesion molecules intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) (Fig. 3.3). Exposure to NPs alone did not affect expression of these inflammatory mediators. Treatment with PCB153 alone significantly increased mRNA levels of TNF- α , IL-1 β , and ICAM-1 but not VCAM-1. Importantly, exposure to PCB153-NPs resulted in significant elevation of mRNA of all proinflammatory mediators assessed in the present study as compared to both controls and PCB153 alone treated mice.

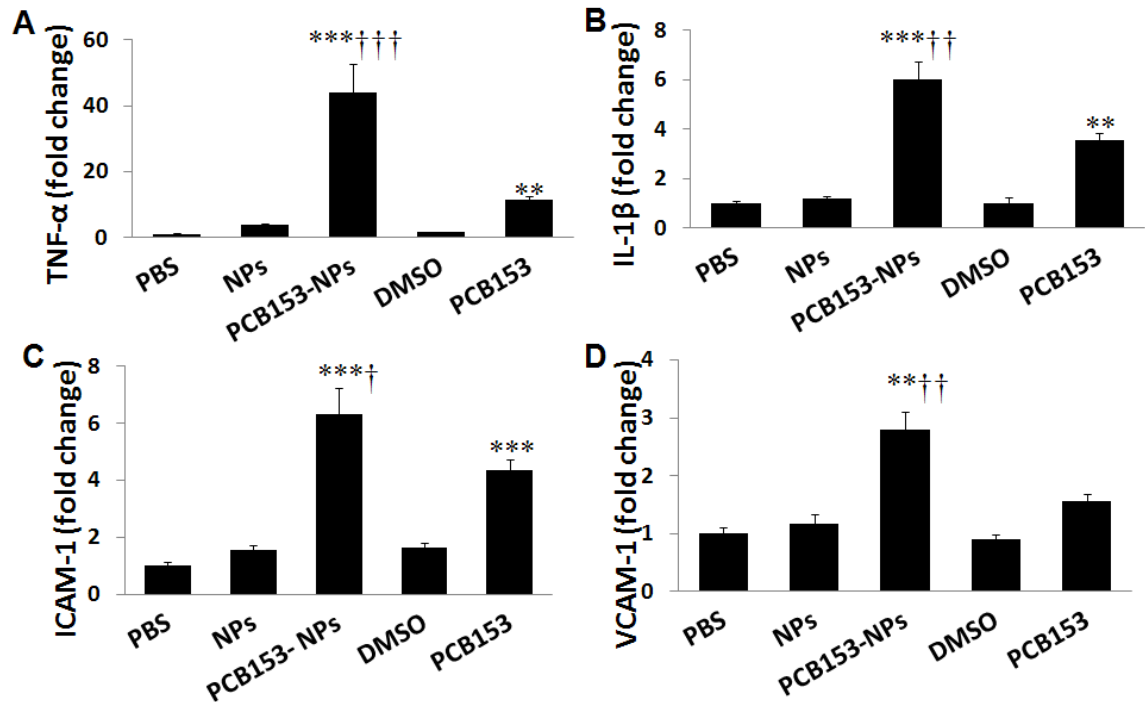


Figure 3.3. Exposure to PCB153-NPs potentiates inflammatory responses in brain capillaries. Mice were treated as in Figure 3.1, followed by brain microvessel isolation. mRNA levels of TNF- α (A), IL-1 β (B), ICAM-1 (C), and VCAM-1 (D) were determined by real-time PCR. Results are mean \pm SEM, n=6. *Significantly different as compared to control groups at **p<0.01 or ***p<0.001. †Results in the PCB153-NPs group are statistically different from those in the PCB153 group at †p<0.05, ††p<0.01, or †††p<0.001.

Exposure to PCB153-NPs induces transcapillary migration of monocytes

Because disruption of the barrier function of the brain endothelium is associated with migration of inflammatory cells into the brain, we evaluated these effects in mice exposed to PCB153 and/or NPs. Mice were injected through the ICA with mouse-derived monocytic J744.1 cells labeled with CFDA-SE. Brain slices were also stained for claudin-5 to visualize brain microvessels. Figure 3.4A indicates enhanced numbers of monocytic clusters in brain microvessels of mice treated with PCB153-NPs as compared to animals exposed to PCB153 alone. No attachment of labeled monocytes was detected in NP- or vehicle-treated animals.

To further confirm these effects, *in vitro* studies were performed using human brain endothelial cells co-cultured with human monocytic THP-1 cells. As shown in Fig. 3.4B, treatment with PCB153-NPs, but not with PCB153 or NPs, enhanced transmigration of THP-1 cells.

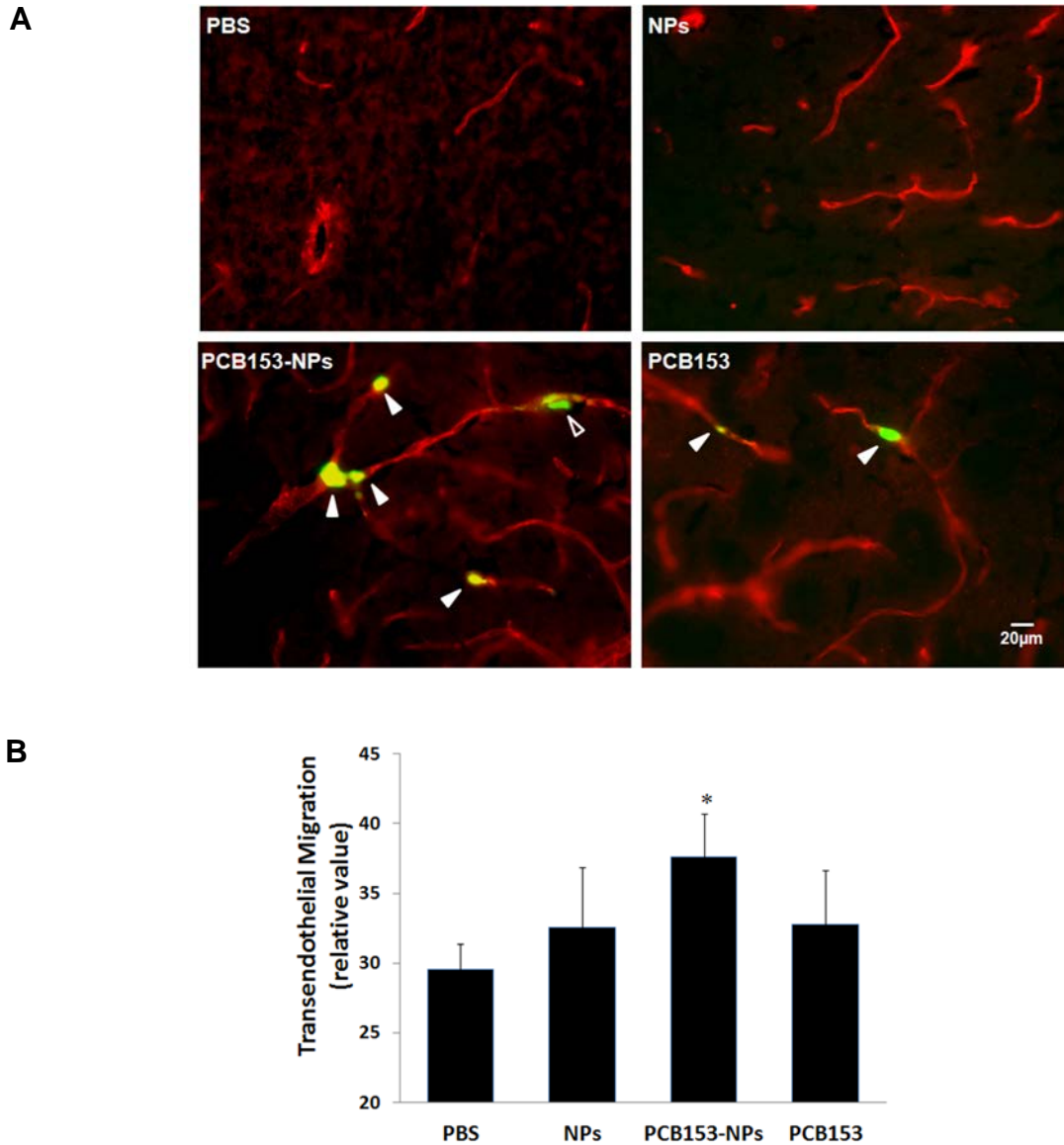


Figure 3.4. Treatment with PCB153-NPs enhances monocyte transmigration. (A) Mice were exposed to vehicle, PCB153, and/or NPs as in Figure 3.1, followed by injection with monocytic J774.1 cells labeled with CFDA-SE (green) into the internal carotid artery. In addition, brain sections were stained for claudin-5 (red) to visualize the vessels. Closed arrowheads indicate labeled J774.1 cells inside cerebral vessels, while open arrowheads indicate labeled J774.1 cells that appear to be present in the perivascular space. Scale bar =20 μ m. (B) Confluent hCMEC/D3 cells cultured on Transwell inserts were treated with PCB153-NPs (PCB153, 1.6 μ M; NPs, 2.08×10^5), PCB153 (1.6 μ M), NPs (2.08×10^5), or vehicle for 24 h. THP-1 monocytic cells were labeled with calcein AM, added on the top of endothelial monolayers, and their transendothelial migration was assessed 4 h later. Data are mean \pm standard deviation (SD), n=6. *Significantly different as compared to control groups at $p < 0.05$.

Exposure to PCB153-NPs potentiates brain injury in the experimental stroke model

We next investigated how binding to NPs can affect toxicity of PCB153 in an experimental stroke model based on middle cerebral artery occlusion (MCAO). Mice were injected with vehicle, PCB153 and/or NPs, into cerebral circulation. Twenty four hours later, the animals were subjected to a 40 min MCAO, followed by a 24 h reperfusion. The infarct volume was evaluated using TTC staining. As indicated in Fig. 3.5, exposure to PCB153-NPs significantly increased the infarct volume induced by ischemia/reperfusion as compared to all other treatment groups.

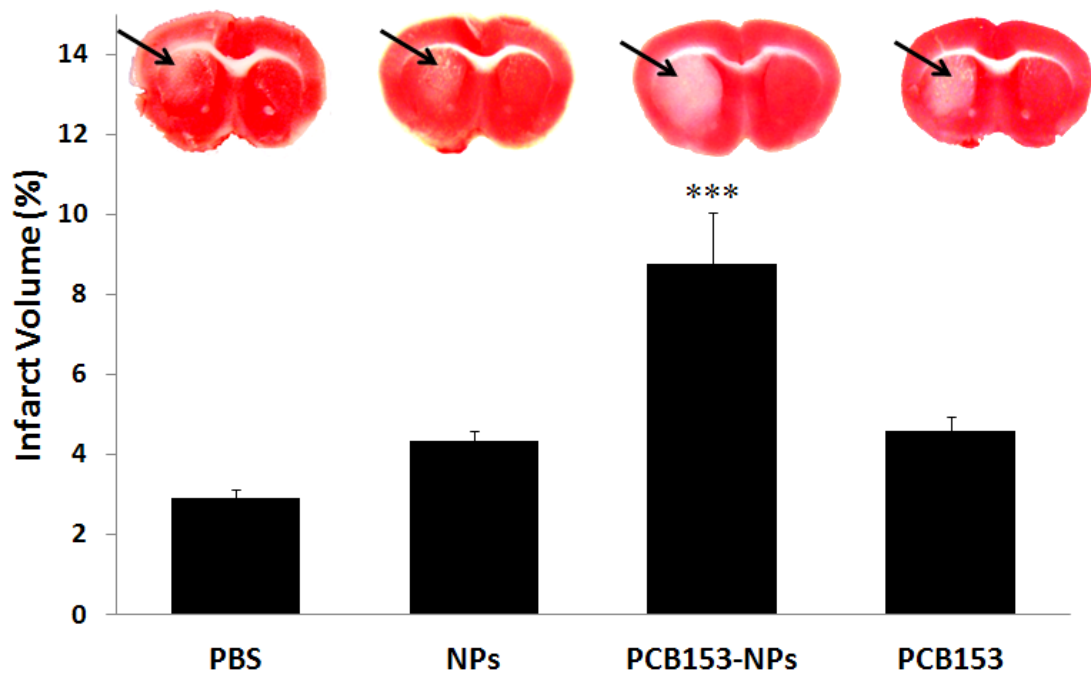


Figure 3.5. Exposure to PCB153-NPs increases the infarct volume in the experimental stroke model. Mice were treated as in Figure 3.1 and subjected to a 40 min MCAO, followed by a 24 h reperfusion, and staining with 2,3,5-triphenyltetrazolium chloride (TTC) to visualize viable tissue. Unstained area (arrows) corresponds to damaged brain tissue. Quantified results of negative TTC staining are depicted in the form of bar graphs. Results are mean \pm SEM, n=5–7. ***Significantly different as compared to other groups at $p<0.001$.

Long-term exposure to PCB153-NPs stimulates accumulation of PCB153 in adipose tissue and potentiates leukocyte attachment to the cerebral vessels

In the final series of experiments, we changed the route of exposure and evaluated the effects of chronic (30 days) oral exposure to PCB153 and/or PCB153-NPs. Treatment with PCB153-NPs resulted in a significantly higher PCB153 accumulation in adipose tissue collected from abdominal cavity as compared to exposure to PCB153 alone (Fig. 3.6A). Importantly, a chronic exposure to PCB153-NPs resulted in enhanced aggregation of leukocytes in brain vessels and perivascular space as visualized through cranial window (Fig. 3.6B). In contrast, chronic treatment with subtoxic levels PCB153 alone did not affect leukocyte aggregation in cerebral vessels.

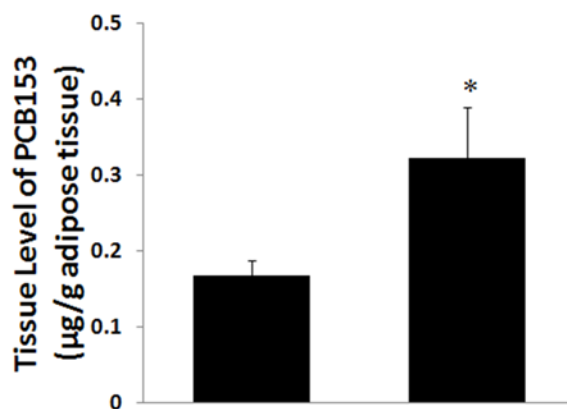
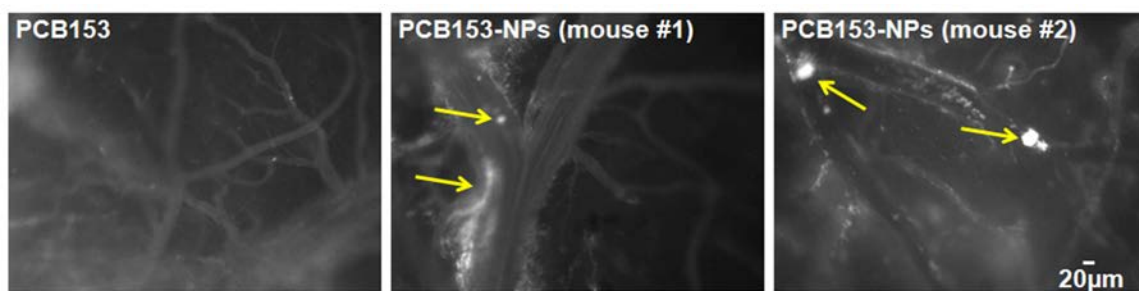
A**B**

Figure 3.6. Long-term exposure to PCB153-NPs potentiates PCB153 adipose accumulation and leukocyte attachment to cerebral vessels. (A) Mice were exposed orally to PCB153-NPs (5 ng PCB153/g body weight bound to 1.04×10^5 silica NPs), the equimolar dose of PCB153, or the appropriate vehicles for 30 days. PCB153 levels were assessed in adipose tissue. Results are mean \pm SEM, n=5–6. *Significantly different as compared to other groups at $p < 0.05$. (B) Mice were exposed as in (A), followed by installation of the cranial window. Circulating leukocytes were fluorescently labeled by i.p. injection with rhodamine 6 G and the interactions of labeled leukocytes with the brain endothelium were detected via cranial window under fluorescent microscope. Arrows indicate leukocytes inside the cerebral vessels that appear to be attached to the brain endothelium. Scale bar=20 μ m.

5. Discussion

The transport modes of PCBs in the environment are multifarious, including binding of environmental PCBs to particulate matter (PM) [274]. In the current study, we hypothesized that such a binding may change toxicological properties of PCBs and influence risk assessment. To address this hypothesis, we evaluated cerebrovascular toxicity of PCB153 bound to silica NPs. The choice of PCB153 was based on the fact that it is one of the most prominent PCB congeners in the environment. Moreover, ortho-substituted PCBs, such as PCB153, accumulate in the CNS [135, 258]. PM collected and analyzed in environmental samples is physically and chemically complex, making analyses of the biological effects of air pollution challenging. In contrast, engineered NPs are mono-dispersed and chemically pure. We used silica NPs as PCB153 carrier due to abundance of silica in the environment. Synthetic silica NPs, being chemically inert, are generally regarded as safe [275] and have been approved for use as food or animal-feed ingredient as well as in diagnostic and biomedical research [276]. Importantly, silica NPs employed in the present study were in the similar size range as particles present in the environment.

Once deposited, the fate of NPs depends on their chemical structure and targeted organs. NPs can reach the CNS through the sensory nerves embedded in the airway epithelia [149], and/or directly through the BBB [150, 151]. While specific NPs can be biodegraded and/or cleared from tissues via the gastrointestinal tract, lymphatic system, and blood circulation, we demonstrated that NPs, such as nano-alumina can still be detected in the brain several days after the initial exposure, causing long lasting effects [277].

Results of the present study indicate that animals exposed to PCB153 bound to silica NPs exhibit a dramatic increase in BBB permeability and decrease of TJ protein expression, effects that are not observed in animals exposed to an equal amount of PCB153 alone. Although specific mechanisms of PCB153-NP-induced disruption of BBB integrity are not known, redox-responsive reactions might be involved in this event. The brain is an organ that is highly vulnerable to oxidative stress due to its high content of iron and lipids, high oxygen consumption, and relatively low capacity of antioxidant

defenses [144]. Such properties make the brain susceptible to PCB-induced injury, as PCBs are recognized as potent inducers of oxidative stress [141, 278]. In fact, a recent study published by our group indicated that PCB153 can induce cellular oxidation acting through the NADPH oxidase complex [143]. Moreover, exposure to particulate matter can also induce production of reactive oxygen species (ROS) in rat brain capillaries, resulting in alterations of expression of TJ proteins [178]. An increase in oxidative stress may provide a common trigger for downstream events that regulate BBB integrity. For example, oxidative stress-induced alterations of TJ proteins may act through Ras and Rho redox responsive elements [279]. Upon activation, Rho phosphorylates downstream molecules such as Rho kinase (ROCK), which then results in increased myosin light chain (MLC) phosphorylation via inhibition of MLC phosphatases [280]. Oxidative stress can also lead to the elevation of chemokine receptors at the brain endothelium, further contributing to MLC phosphorylation through the activation of myosin light chain kinase (MLCK) [281]. MLCK can modulate actin structure and phosphorylate TJ proteins, which results in the cytoskeletal reorganization. Under conditions of oxidative stress, activated protein tyrosine kinases (PTKs) may also trigger activation of matrix metalloproteinases (MMPs), which can degrade the endothelium basement membrane, leading to the disruption of the BBB [34].

Recent reports from our laboratory implicate the brain endothelium as an important target of vascular toxicity of PCBs. Indeed, exposure to specific PCB congeners, including PCB153, stimulates induction of adhesion molecules and induces leukocyte adhesion [143, 282]. These effects are important because inflammatory responses involving cerebral vessels contribute to the development of neuroinflammation, constitute the risk of onset of stroke, and potentiate tissue damage in brain ischemia/reperfusion [71]. Upregulation of ICAM-1 and VCAM-1 expression by brain endothelial cells has also been associated with multiple sclerosis, encephalitis, and other inflammatory conditions in the brain [283]. Therefore, it is of significant clinical relevance that exposure to PCB153-NPs results in more pronounced expression of proinflammatory mediators as compared to treatment with PCB153 alone. Overexpression of inflammatory cytokines, such as TNF- α and IL-1 β , can further stimulate production of proinflammatory mediators, impair BBB functions, and recruit

inflammatory cells. Moreover, inflammatory cytokines can induce expression of adhesion molecules on endothelial cells, facilitating the adherence of leukocytes to the brain endothelium. A functional BBB is critical in maintaining the immune-privileged status of the brain. Thus, a strategy targeting inhibition of proinflammatory mediators may help to protect against PCB- or other environment pollutant-induced BBB dysfunction.

Increased expression of adhesion molecules results in enhanced adhesive properties of endothelial cells and stimulation of adhesion of leukocytes to the brain endothelium [234, 284]. Therefore, we investigated the influence of PCB153 and/or NPs on leukocyte adhesion using both in vivo and in vitro models. Visualization of leukocyte-endothelium interaction was accomplished in vivo by detection of labeled leukocytes by fluorescent microscopy via a cranial window. While this method is suitable to study the interactions of leukocytes with brain microvessels, in vitro modeling of transendothelial migration of monocyte allows for quantitative comparison among different treatments.

Consistent with the effects on expression of proinflammatory mediators, both acute and chronic administration of PCB153-NPs significantly upregulate monocyte attachment to the brain endothelium, with PCB153 having only a moderate effect. These results indicate that brain microvessels can recognize and respond to PCB153-NPs by producing proinflammatory molecules. This response may serve as a proinflammatory sensor and ultimately distribute ROS and cytokines, further contributing to the CNS pathology.

Enhanced cerebrovascular toxicity of PCB153-NPs, as compared to PCB153 alone, may result from more effective bioaccumulation of these toxicants. The properties of NPs as effective carriers across the BBB are well described and have been used for drug delivery into the brain [285]. Another possibility is lower excretion rate of PCB153-NPs due to the longer retention of NPs [286]. Indeed, we observed a higher PCB153 body burden in animals chronically exposed to PCB153-NPs than in PCB153 alone. These measurements were performed in adipose tissue to reflect lifetime PCB bioaccumulation [287]. Because chronic exposure to PCB153 and/or NPs was administered, effects of PCB153 and NPs on intestinal epithelium might also contribute to increased PCB153 absorption into the blood stream. In fact, we recently demonstrated

that oral exposure to PCBs can disrupt TJ proteins in small intestine and increase intestinal permeability [278].

Epidemiologic studies and laboratory evidence demonstrate that exposure to PCBs may enhance the risk for stroke and other vascular disorders [14, 130]. Therefore, one aim of the present study was to investigate the effects of PCB153 and/or NPs on the development and outcome of stroke. We detected a larger stroke volume in mice exposed to PCB153-NPs, as compared to other treatment groups, which was consistent with the effects of these toxicants on TJ protein expression and proinflammatory responses. Indeed, endothelial activation, proinflammatory and prothrombotic interactions between the vessel wall and circulating blood constituents are known risk factors triggering the onset of stroke [288].

In summary, we demonstrate for the first time that binding of PCB153 to silica NPs can increase cerebrovascular toxicity of these toxicants by enhancing proinflammatory responses and disruption of the BBB integrity. These effects appear to predispose mice to stroke and potentiate brain injury associated with ischemia/reperfusion. Our results also emphasize the importance of risk assessment for NPs by demonstrating that even chemically inert silica NPs can markedly influence toxicity of other environmental chemicals, such as PCBs.

Chapter four: TLR4 signaling is involved in brain vascular toxicity of PCB153 bound to nanoparticles

1. Synopsis

PCBs bind to environmental particles; however, potential toxicity exhibited by such complexes is not well understood. The aim of the present study was to study the hypothesis that assembling onto nanoparticles can influence the PCB153-induced brain endothelial toxicity via interaction with the toll-like receptor 4 (TLR4). To address this hypothesis, TLR4-deficient and wild type control mice (males, 10 week old) were exposed to PCB153 (5 ng/g body weight) bound to chemically inert silica nanoparticles (PCB153-NPs), PCB153 alone, silica nanoparticles (NPs; diameter, 20 nm), or vehicle. Selected animals were also subjected to 40 min ischemia, followed by a 24 h reperfusion. As compared to exposure to PCB153 alone, treatment with PCB153-NP potentiated the brain infarct volume in control mice. Importantly, this effect was attenuated in TLR4-deficient mice. Similarly, PCB153-NP-induced proinflammatory responses and disruption of tight junction integrity were less pronounced in TLR4-deficient mice as compared to control animals. Additional *in vitro* experiments revealed that TLR4 mediates toxicity of PCB153-NP via recruitment of tumor necrosis factor-associated factor 6 (TRAF6). The results of current study indicate that binding of PCBs to seemingly inert nanoparticles increase their cerebrovascular toxicity and suggest that targeting the TLR4/TRAF6 signaling may protect against these effects.

2. Introduction

Environmental exposure to polychlorinated biphenyls (PCBs) is an ongoing environmental problem. Because of their chemical stability, slow degradation rate, and high tendency to bioaccumulate in the food chain, PCBs are among the most persistent and widespread organic pollutants [253]. The fate and transport of PCBs are associated with the specific structure of individual PCB congeners. PCBs are readily adsorbed onto particles, such as atmospheric particulates, soil, and sediments. Recent publications have reported PCBs bound to particles ranging from 0.95 μm to 1.5 μm in ambient air [260-262].

Exposure to PCBs has been linked to various adverse health effects in humans. Recent reports from a PCB contaminated site located in Anniston, AL indicated that serum PCB levels are highly associated with increased prevalence of diabetes [256] and hypertension [132]. These diseases are considered to be risk factors for stroke. In fact, an increase in the incidence of stroke was observed in people exposed to PCBs [289, 290] and living in proximity to PCB hazardous wastes [14]. These observations are important because stroke is one of the leading causes of death worldwide.

A functional blood-brain barrier (BBB) is the key element for the homeostasis of the central nervous system (CNS). The BBB consists of highly specialized brain endothelial cells that are characterized by the unique phenotype of intercellular tight junctions (TJs) and numerous polarized transport systems [51]. Disruption of TJ proteins is often observed during acute and chronic diseases of the CNS, including stroke [291]. Our research group reported that oral administration of selective PCB congeners resulted in accumulation of PCBs in brain tissue and increased permeability of the BBB [135, 144, 282]. Highly chlorinated *ortho*-PCBs preferentially accumulate in brain tissue and are associated with several CNS diseases, such as Parkinson's disease [292] or developmental alterations [293]. The most representative *ortho*-PCB congener is 2,2',4,4',5,5'-hexachlorobiphenyl (PCB153), which is commonly detected in human [259, 294] and in environmental samples, including atmospheric particulates [295, 296]. We hypothesize that binding of PCB153 to nanoparticles (NPs) can influence their toxic properties. However, the mechanisms by which PCB153-NP complexes are sensed and transduced via cellular signaling are largely unknown.

Biological systems universally respond to various stimuli of environmental signals by using evolutionarily conserved mechanisms [297, 298]. One such example is toll-like receptors (TLRs), which recognize and respond to an expansive variety of environmental and pathogen associated molecular stimuli [299, 300]. TLRs are widely expressed in various cell types in the brain, including microglia, astrocytes, neurons, and endothelial cells [301, 302]. Recent evidence indicates that TLR4, the first characterized of mammalian TLRs, may play a vital role in ischemia/reperfusion injury [180, 303].

In the present study, we hypothesize that exposure to PCB153 assembled onto nanoparticles contributes to the development of stroke by disruption of the integrity of the cerebral endothelium and induction of proinflammatory responses through stimulation of TLR4 signaling. The results of the present study support this notion and indicate that targeting of the TLR4/tumor necrosis factor-associated factor 6 (TRAF6) signaling can protect against cerebrovascular toxicity of PCB153-NP complexes.

3. Materials and Methods

Materials

2,2',4,4',5,5'-hexachlorobiphenyl (PCB153) congener was purchased from AccuStandard (New Haven, CT) and silica NPs from NanoAmor (Houston, TX). Characterization of NPs and construction of silica NPs coated with PCB153 were described in our previous study [293]. Briefly, silica NPs (80 mg) and PCB153 (10 mg) were dispersed in acetone and sonicated to prevent aggregation. The highly hydrophobic surface character of PCB153 and silica NPs allows them to interact with each other based on electrostatic attraction. After evaporation of acetone, the particles were resuspended in phosphate buffered saline (PBS) or cell culture medium, sonicated, and centrifuged at 12,000 rpm for 5 min. The supernatant containing PCB153-NPs was then collected to analyze PCB153 levels by gas chromatography/mass spectrometry (GC/MS). The hydrodynamic size distribution and the amounts of PCB153-NPs were monitored by dynamic light scattering (DLS) and atomic force microscope (AFM), respectively. Control NPs were prepared using the same procedure, without adding PCB153. All treatment factors were tested for possible endotoxin contamination using the LAL chromogenic endotoxin quantitation kit (Thermo Scientific Pierce, Rockford, IL). The

levels of endotoxin in all preparations were below the detection limit, indicating no contamination.

Experimental groups and surgical procedures

All experimental procedures and protocols were approved by the National Institutes of Health Guide for the Care and Use of Laboratory Animals. C3H/HeJ mice contain a point mutation in the TLR4 gene and are TLR4-deficient, whereas C3H/HeouJ mice express normal TLR4 activity and were used as controls. Mice (males, 10-12 weeks old; Jackson Laboratories) were infused with a) PCB153 bound to nanoparticles (PCB153-NPs), b) PCB153 dissolved in 0.01% DMSO (PCB153), c) nanoparticles (NPs) alone, or d) vehicle (PBS). PCB153 was administered in the amount of 5 ng/g body weight. All infusions were performed through the internal carotid artery (ICA) using a surgical technique standardized by our research group [270] for selective drug delivery into the brain vasculature.

Transient focal cerebral ischemia was induced by a 40 min occlusion of the middle cerebral artery (MCA), following a 24 h reperfusion as described previously [304]. In anesthetized mice, a 6-0 surgical nylon suture coated with silicon (Doccol, Redlands, CA) was advanced through the left common carotid artery and up to the ICA to block the origin of the MCA. After occlusion for 40 min, reperfusion was initiated by removing the suture to restore the blood flow.

Assessment of the infarct volume

The mouse brain was removed and sectioned into 7 coronal slices with 1 mm thickness from the frontal pole to the occipital pole using a coronal acrylic matrice (Braintree Sci., Braintree, MA). The brain slices were then stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC) at 37 °C for 20 min. The viable brain tissue was stained in red, whereas the infarcted area appeared unstained. The infarct size and volume were calculated using ImageJ software as previously described [271].

Brain microvessel isolation

Isolation of brain microvessels was performed as described previously [144]. After removing meninges and choroids plexus, brain tissue was homogenized in ice-cold buffer containing 103 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 15 mM HEPES, 25 mM NaHCO₃, 10 mM glucose, 1 mM Na pyruvate, 10 g/L dextran and protease inhibitor cocktail tablets (Roche Diagnostics, Indianapolis, IN). The homogenates were mixed with 26% dextran and centrifuged at $5,800 \times g$ at 4 °C for 20 min. The collected pellets were resuspended in ice-cold buffer and filtered through a 70 µm cell strainer (BD Biosciences, San Jose, CA). Filtered samples were re-pelleted by centrifugation, followed by either resuspension in 150 µL of 6 M urea lysis buffer for Western blot analyses, or resuspension in 200 µL of TRIZOL (Invitrogen, Carlsbad, CA) for total RNA extraction.

Cell cultures, treatment factors, and gene silencing

Human brain endothelial cells (hCMEC/D3 cell line) were cultured as previously described [143]. Confluent cultures were exposed to PCB153-NPs, NPs, PCB153 alone, or vehicle for 24 h. In cell culture experiments, PCB153 was used in subtoxic concentration of 1.6 µM, which is lower than the levels reported in humans acutely exposed to PCBs [305, 306]. In selected experiments, cultured cells were treated with 10 µM CLI095, a pharmacological inhibitor of TLR4, which blocks the signaling mediated by the intracellular domain of TLR4.

Cultured cells at 70-80% confluency were transfected with 60 nM of control or TRAF6 specific siRNA (Applied Biosystems, Carlsbad, CA) using GeneSilencer (Genlantis, San Diego, CA). The cells were incubated with transfection mixtures for 24 h and allowed to recover in complete medium for 48 h before exposure to PCB153 and/or NPs.

Immunoblotting and immunoprecipitation

Immunoblotting was performed with either whole cell lysates (30 µg protein per sample) prepared in RIPA lysis buffer (50 mM Tris-HCl pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, and 1 mM EDTA) or lysed mouse brain microvessels (50 µg protein per sample). Protein samples were separated on SDS-

polyacrylamide gel, blotted onto polyvinyl difluoride membranes (Bio-Rad Laboratories, Hercules, CA), and incubated with the respective antibodies. Anti-occludin and anti-claudin-5 antibodies were from Invitrogen, anti-TLR4 antibody from Santa Cruz Biotechnology (Santa Cruz, CA), anti-actin antibody from Sigma, and all secondary antibodies from Cell Signaling Technology (Danvers, MA). For visualization of detected proteins, immunoblots were analyzed using an ECL Western blot detection kit (GE Healthcare Life Sciences, Piscataway, NJ) and proteins of interest were semi-quantitated with ImageJ software.

Immunoprecipitation of TRAF6 was performed using 800 µg of protein extracted from whole cell lysate. Samples were incubated with 1 µg of anti-TRAF6 antibody (Cell Signaling Technology) overnight at 4 °C. Next day, 30 µL of Protein A/G Plus Agarose (Thermo Scientific Pierce, Rockford, IL) was added to each sample and immunoprecipitation was performed for 2 h at 4 °C. Bound proteins were eluted by boiling in SDS sample buffer for 5 min and analyzed on SDS-polyacrylamide gel.

Real-time RT-PCR

Total RNA was extracted from freshly isolated microvessels using TRIZOL reagent (Invitrogen) according to the manufacturer's instructions with an additional chloroform extraction, phase separation, and an extra wash in 70% ethanol. Then, 1 µg of RNA was reverse-transcribed using the Reverse Transcription System (Promega, Madison, WI) and 3 µL of final RT product was used for PCR amplification. Taqman Universal PCR Master Mix, pre-developed primer pairs and probes were purchased from Applied Biosystems (Foster City, CA). The following thermocycling conditions were employed: 95 °C for 10 min, followed by 95 °C for 15 sec, and 60 °C for 60 sec (for up to 40 cycles). Expression of mRNA was calculated and analyzed by the comparative C_T method as described [221].

ELISA

The levels of cytokines and chemokines in cell culture media were determined using Multi-Analyte ELISArray kit (Qiagen, Valencia, CA). Briefly, 50 µL aliquots of culture media were added into individual wells of the ELISArray kit and incubated at

room temperature for 2 h. After the plate was washed three times with washing buffer, 50 μ L of biotin-conjugated anti-IL-6, anti- CXCL-8, anti-CCL-2, and anti-CCL-5 antibodies were added into indicated wells and incubated at temperature for 1 h. Then, the plate was washed three times and avidin-conjugated horseradish peroxidase was added to each well for 30 min incubation at room temperature, followed by four washings with the washing buffer. After 15 min incubation with development solution, stop solution was added to each well and the absorbance was measured at 450 nm using SpectraMax 190 absorbance microplate reader (Molecular Devices, Sunnyvale, CA). The standard curve was generated using antigen standard of each target protein at the concentrations between 0 to 200 pg/mL.

Statistical analysis

Statistical analysis was completed using SigmaPlot 12.0 (Systat Software, San Jose, CA). One-way or Two-way ANOVA followed by Holm-Sidak *post hoc* test was used to compare mean responses among the treatments. A statistical probability of $p < 0.05$ was considered significant.

4. Results

TLR4 deficiency diminishes PCB153-NP-induced enhancement of infarct volume and disruption of the BBB integrity

To test the hypothesis that PCB153-NPs potentiate the ischemic injury through activation of TLR4, mice with a point mutation in the TLR4 gene (C3H/HeJ) and mice expressing normal TLR4 activity (C3H/HeouJ) were employed. As indicated in Figure 4.1, exposure to PCB153-NPs significantly increased the infarct volume in the control mice as compared to treatment with vehicle (PBS), NPs, or PCB153 alone. However, TLR4-deficient mice infused with PCB153-NPs showed significantly smaller infarct volume as compared to control mice.

Disruption of TJs is a typical event during cerebral ischemia. Therefore, we evaluated the effects of PCB153 and/or NPs on expression of transmembrane TJ proteins, such as occludin and claudin-5 as well as TJ-associated protein ZO-1 both in animals and cell cultures of brain endothelial cells. Exposure to PCB153-NPs but not to PCB153 or

NPs alone resulted in a decrease in claudin-5 and ZO-1 levels in mice with normal TLR4 expression, whereas deficiency of TLR4 diminished the effect (Figure 4.2A). Consistent with these results, levels of occludin and claudin-5 were also markedly reduced following exposure to PCB153-NPs in brain endothelial cells. Importantly, inhibition of TLR4 activity with CLI095 attenuated these effects (Figure 4.2B), further indicating that TLR4 pathways is involved in PCB153-NP-induced alteration of TJ expression.

Overexpression of proinflammatory cytokines, chemokines, and adhesion molecules in the brain is hallmark of neuroinflammation. Therefore, we evaluated the expression levels of proinflammatory cytokines (IL-6 and CXCL-8 [IL-8]), chemokines (CCL-2 and CCL-5 [RANTES]) and adhesion molecule ICAM-1 following exposure to PCB153 and/or NPs in brain microvessels and cultured brain endothelial cells. As shown in Figure 4.3A, mRNA levels of IL-6, CCL-2, CCL-5, and ICAM-1 were significantly elevated in brain capillaries of wild type mice exposed to PCB153-NPs but not to PCB153 or NPs alone. Importantly, deficiency of TLR4 effectively protected against these effects. Inhibition of TLR4 signaling by CLI095 also attenuated PCB153-NP-induced overproduction of IL-6, CXCL-8, CCL-2 and CCL-5 protein levels in cultured human brain endothelial cells (Figure 4.3B).

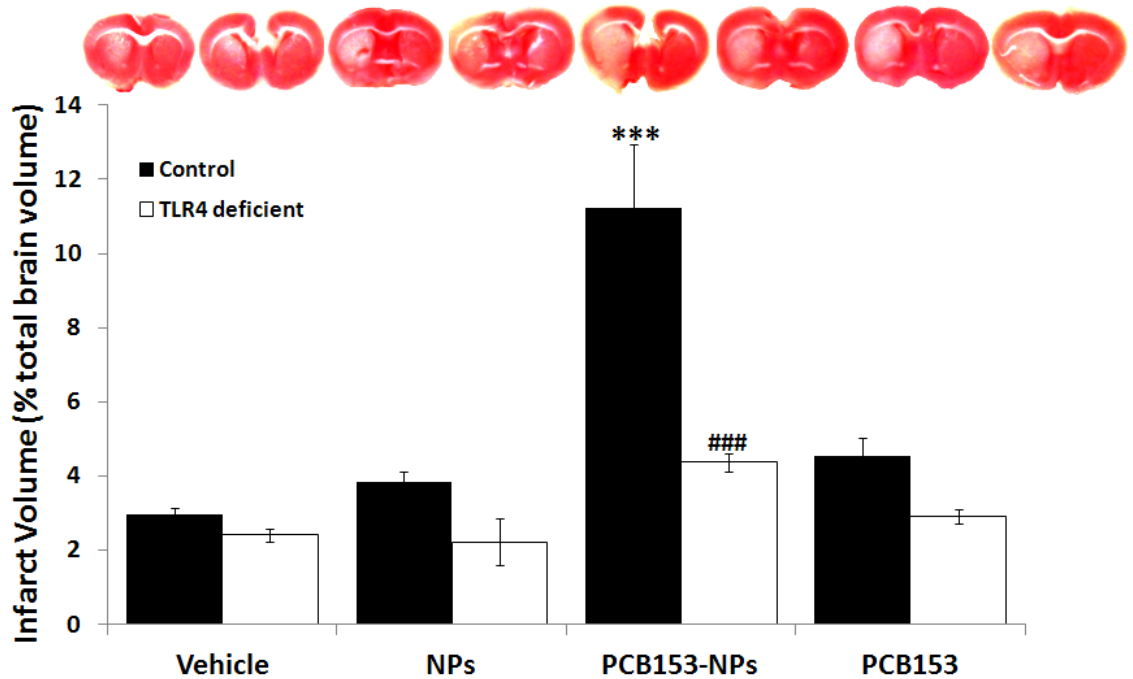
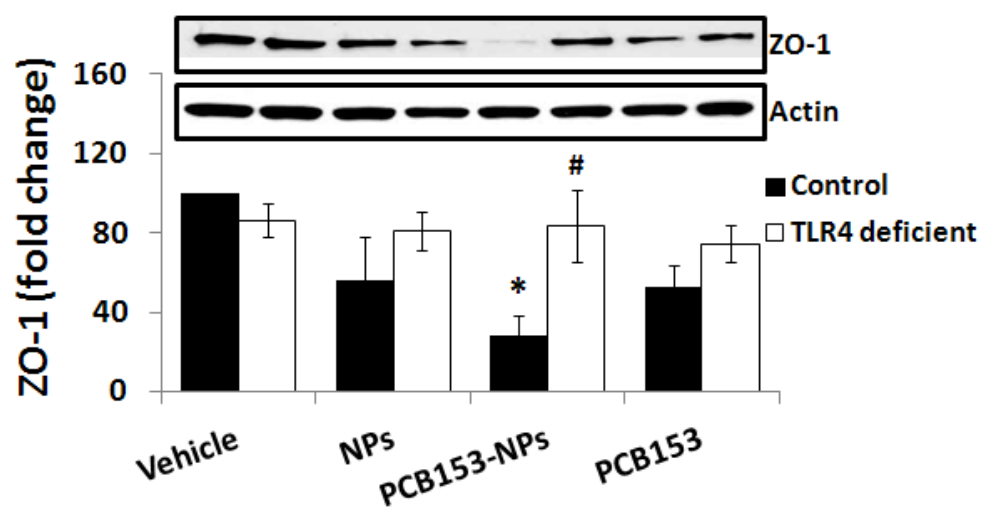
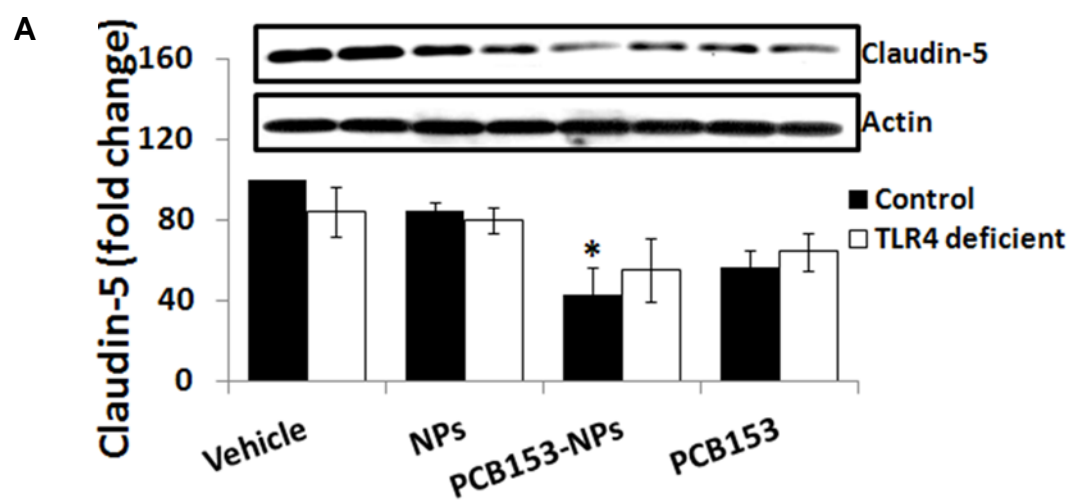


Figure 4.1. PCB153-NP-induced enhancement of infarct volume following ischemia/reperfusion is reduced in TLR4-deficient mice. Mice were exposed to PCB153-NPs (5 ng PCB153/g body weight bound to 1.04×10^5 silica NPs) by infusion into the internal carotid artery (ICA). Control mice were infused with the same amounts of NPs, PCB153 or vehicle (PBS). After 24 h, all animals were subjected to a 40 min occlusion of the middle cerebral artery (MCA), followed by a 24 h reperfusion. The infarct area was detected by 2,3,5-triphenyltetrazolium chloride (TTC) staining and the image illustrates the representative results. The loss of tissue viability is reflected by unstained (white) areas. Quantified results are depicted in a bar graph. Results are means \pm SEM, n=5. Significantly different as compared to vehicle treatment in mice with normal TLR4 expression at ***p<0.001. Results in the TLR4-deficient mice treated with PCB153-NPs are statistically different from those in control animals exposed to PCB153-NPs at ###p<0.001.



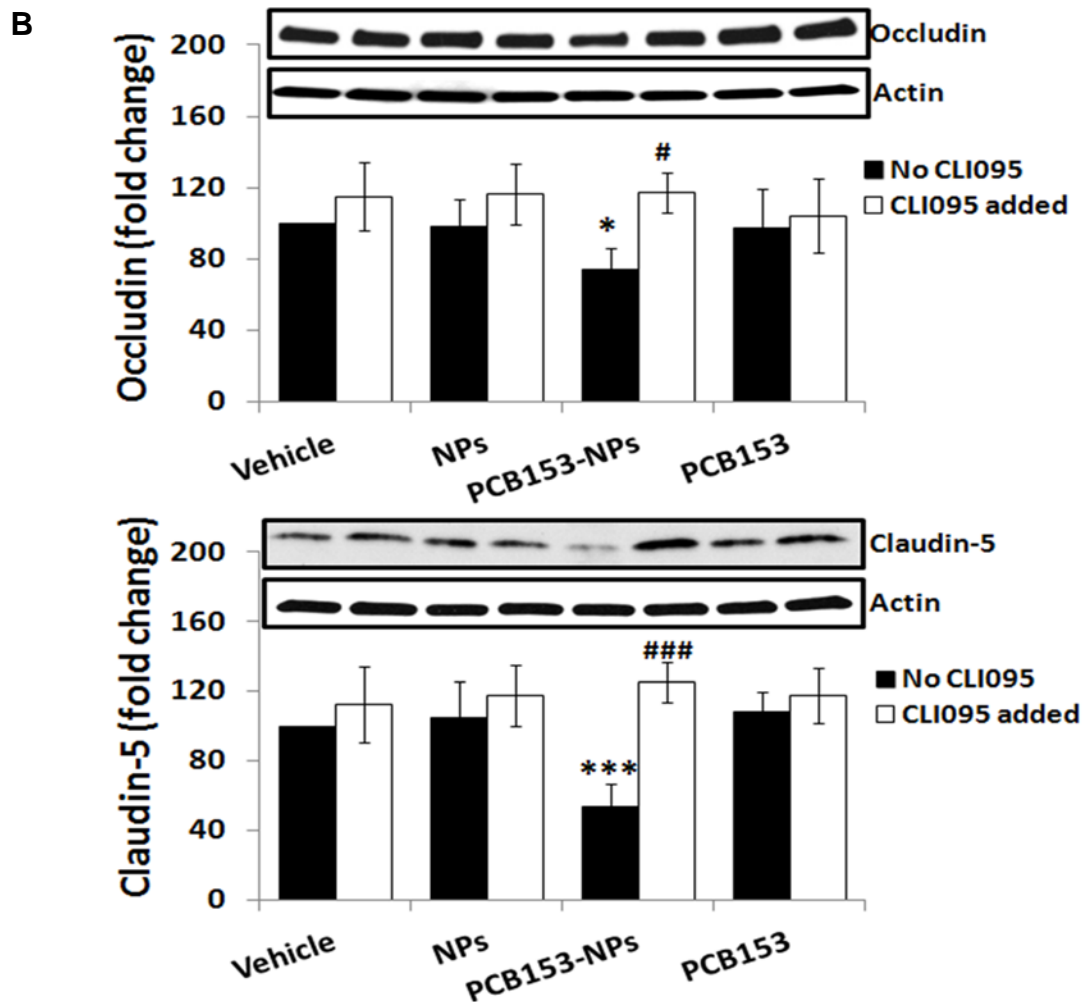
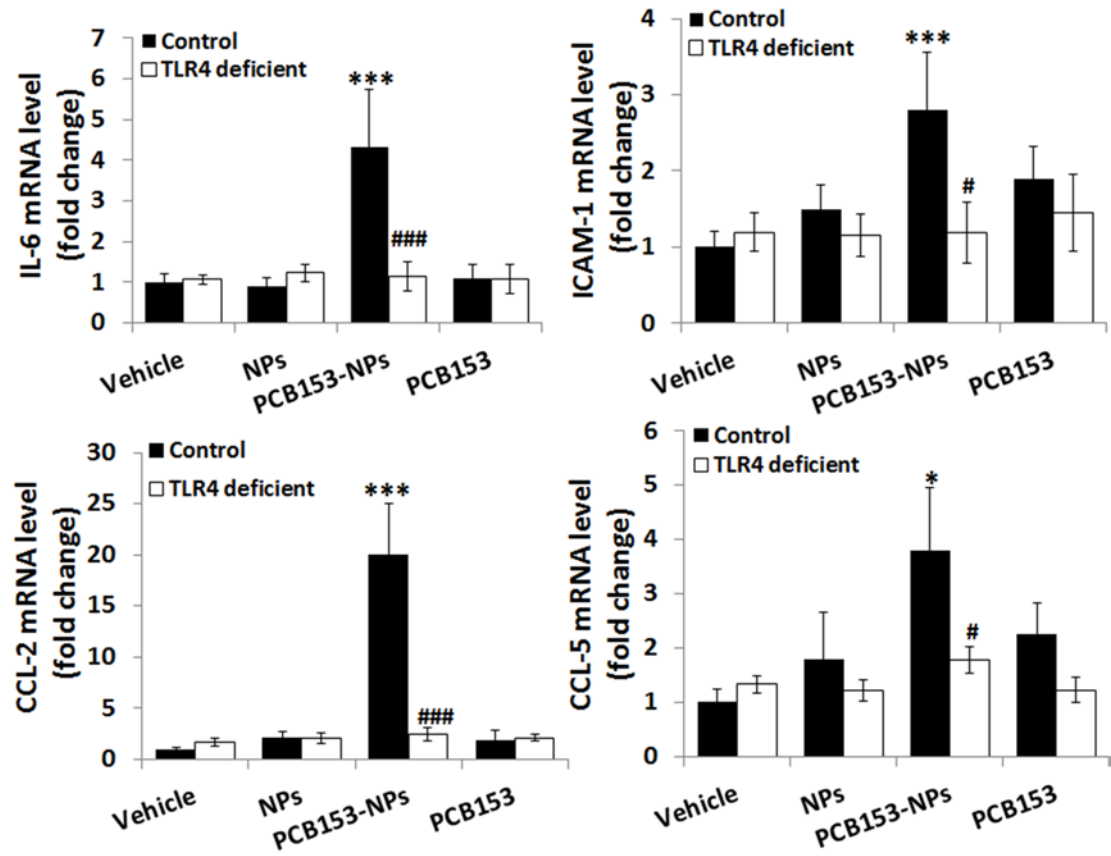


Figure 4.2. TLR4 is involved in PCB153-NP-induced TJ protein disruption. (A) Mice were infused with PCB153-NPs or control treatments as in Figure 4.1. Expression of ZO-1 and claudin-5 was analyzed in brain microvessels isolated from TLR4-deficient or control mice by immunoblotting. Results are means \pm SEM, $n=5$. Significantly different as compared to control treatments in normal mice at $*p<0.05$. Results in the TLR4-deficient mice treated with PCB153-NPs are statistically different from those in control animals exposed to PCB153-NPs at $\#p<0.05$. (B) Confluent brain endothelial cell cultures were treated with PCB153-NPs (PCB153, $1.6 \mu\text{M}$; NPs, 2.08×10^5), or the same amounts of PCB153, NPs, or vehicle for 24 h. Selective cultures were pretreated with TLR4 inhibitor CLI095 ($10 \mu\text{M}$) or vehicle (DMSO, 0.01%) for 1 h, followed by co-exposure to PCB153 and/or NPs for 24 h. TLR4 inhibitor was retained in media throughout PCB153 and/or NPs treatment. Occludin and claudin-5 levels were measured by immunoblotting. Results are means \pm SD, $n=5$. Significantly different as compared to vehicle at $*p<0.05$ or $***p<0.001$. Results in cultures pretreated with CLI095 are statistically different from those in the corresponding cultures without added CLI095 at $\#p<0.05$ or $###p<0.001$.

A



B

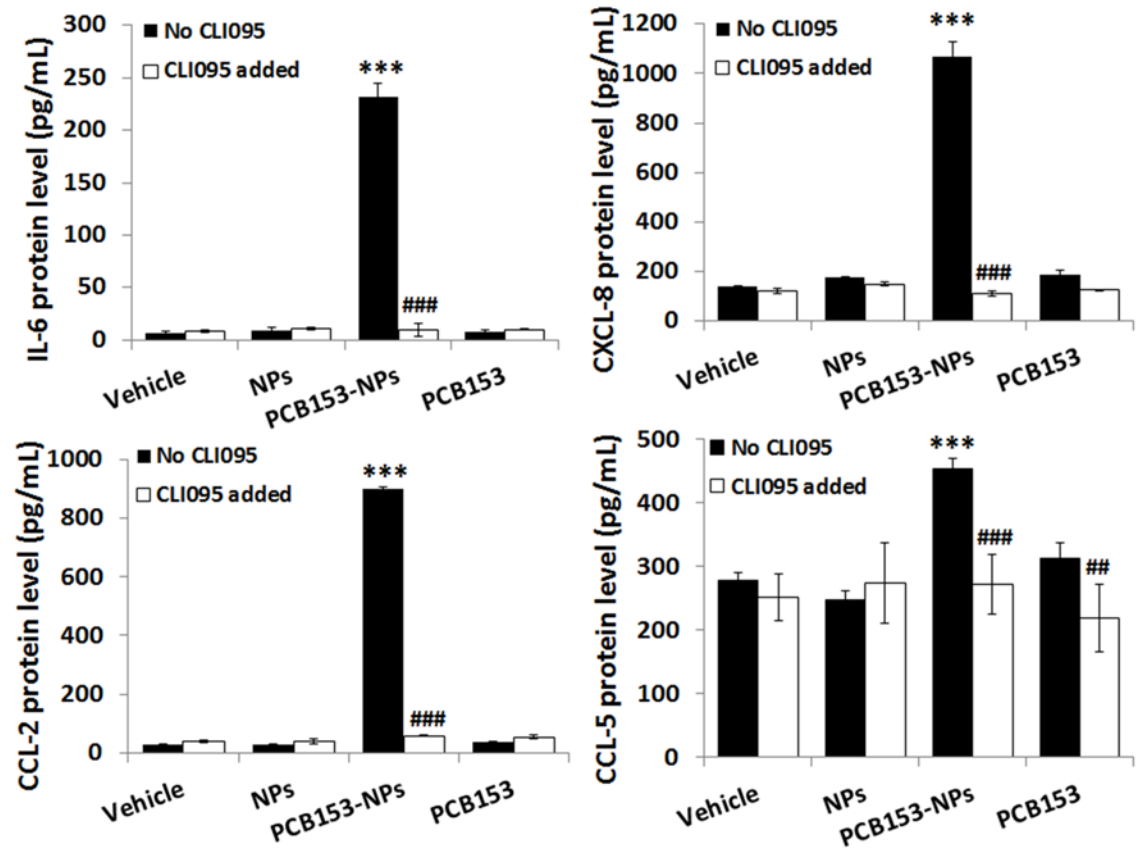


Figure 4.3. Exposure to PCB153-NPs promotes inflammatory responses via TLR4. (A) Mice were treated as in Figure 4.1. mRNA levels of IL-6, ICAM-1, CCL-2 (MCP-1) and CCL-5 (RANTES) were determined in isolated brain microvessels by real-time PCR. Results are means \pm SEM, $n=5$. Significantly different as compared to vehicle treatments in normal mice at $*p<0.05$, or $***p<0.001$. Results in the TLR4-deficient mice treated with PCB153-NPs are statistically different from those in control animals exposed to PCB153-NPs at $\#p<0.05$ or $###p<0.001$. (B) Confluent brain endothelial cells were treated as in Figure 4.2B for 24 h. Protein levels of IL-6, CXCL-8 (IL-8), CCL-2 and CCL-5 were determined by ELISA in cell culture supernatants. Significantly different as compared to vehicle at $***p<0.001$. Results in cultures pretreated with CLI095 are statistically different from those in the corresponding cultures without added CLI095 at $##p<0.01$ or $###p<0.001$.

Exposure to PCB153-NPs induces TRAF6 interaction with TLR4

Upon activation, TLRs recruit adaptor molecules, such as MyD88, which then activate a series of downstream signaling molecules, including TRAF6 [300]. To investigate these events, brain endothelial cells were treated with PCB153-NPs for up to 4 h. Cell lysates were then immunoprecipitated with anti-TRAF6 antibody and probed for TLR4. Figure 4.4A indicates that PCB153-NPs induced a rapid (10 min) but transient recruitment of TRAF6 to TLR4. Treatment with PCB153 alone for 10 min also resulted in binding of TRAF6 to TLR4; however, this effect was less prominent as compared to PCB153-NPs (Figure 4.4B).

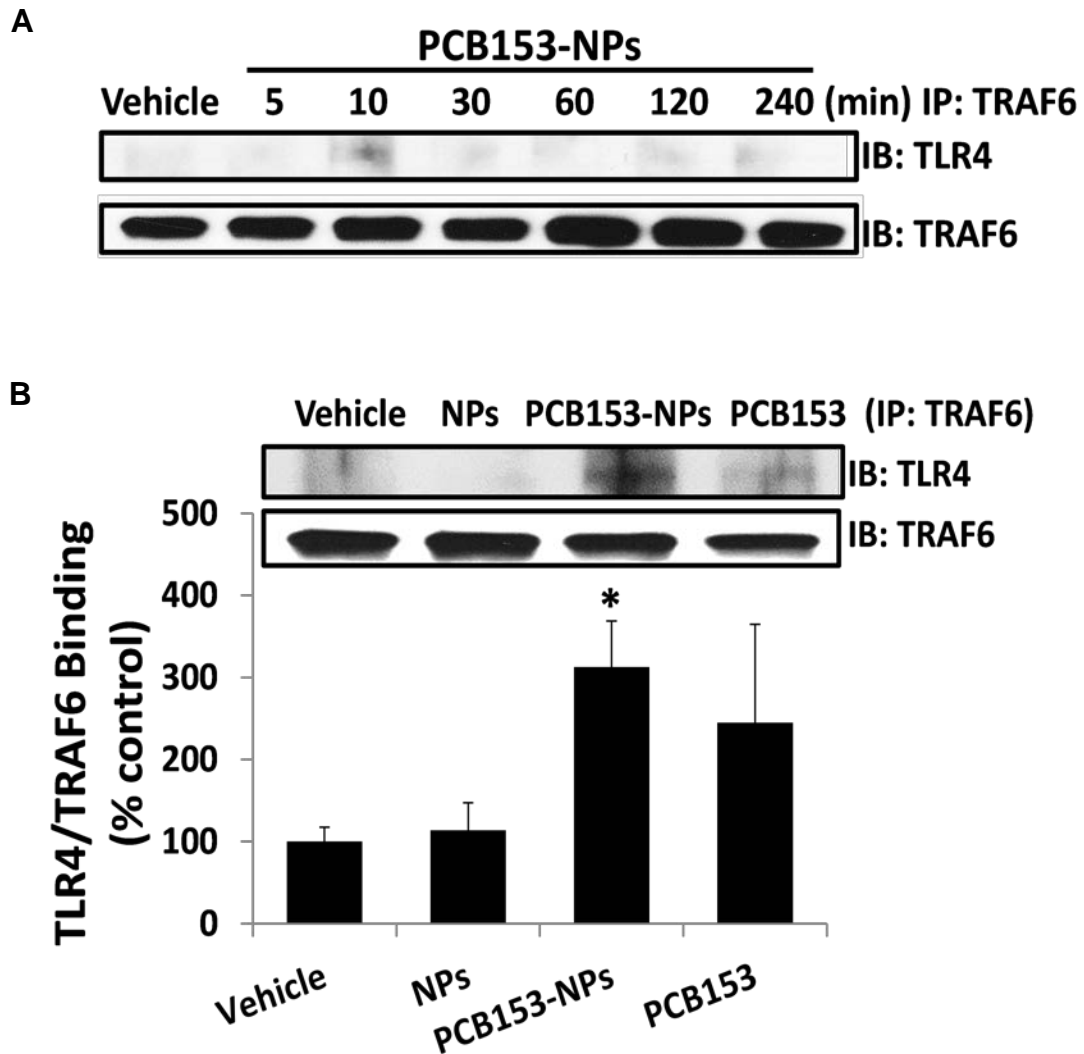


Figure 4.4. Exposure to PCB153-NPs induces TLR4 interaction with TRAF6. (A) Confluent brain endothelial cells were treated with PCB153-NPs for the indicated time. Cellular extracts were immunoprecipitated using anti-TRAF6 antibody, followed by immunoblotting with anti-TLR4 antibody. (B) Confluent brain endothelial cells were exposed to PCB153 and/or NPs for 10 min, followed by determination of TLR4 interaction with TRAF6 as in Figure 4.4A. The blot illustrates the representative data of four independent experiments and the bar graph shows quantified results. Results are the mean \pm SD, n=4. Significantly different as compared to vehicle treatments in normal mice at *p<0.05.

TRAF6 mediates PCB153-NP-induced alterations in TJ protein expression and proinflammatory responses

To investigate the involvement of TRAF6 in PCB153-NP-mediated TJ disruption, expression of TRAF6 in brain endothelial cells was silenced with TRAF6 siRNA (Figure 4.5A), followed by exposure to PCB153 and/or NPs for 24 h. The results indicated that silencing of TRAF6 markedly protected against PCB153-NP-mediated reduction in occludin (Figure 4.5B) and claudin-5 (Figure 4.5C) protein levels.

In the last series of experiments, we investigated the role of TRAF6 in PCB153-NP-stimulated production of inflammatory mediators. Consistent with the results in Figure 3B, exposure to PCB153-NPs significantly increased the production of IL-6, CXCL-8, CCL-2 and CCL-5 in brain endothelial cells transfected with scrambled (control) siRNA. Importantly, silencing of TRAF6 effectively reduced the production of these inflammatory mediators in response to PCB153-NPs (Figure 4.6).

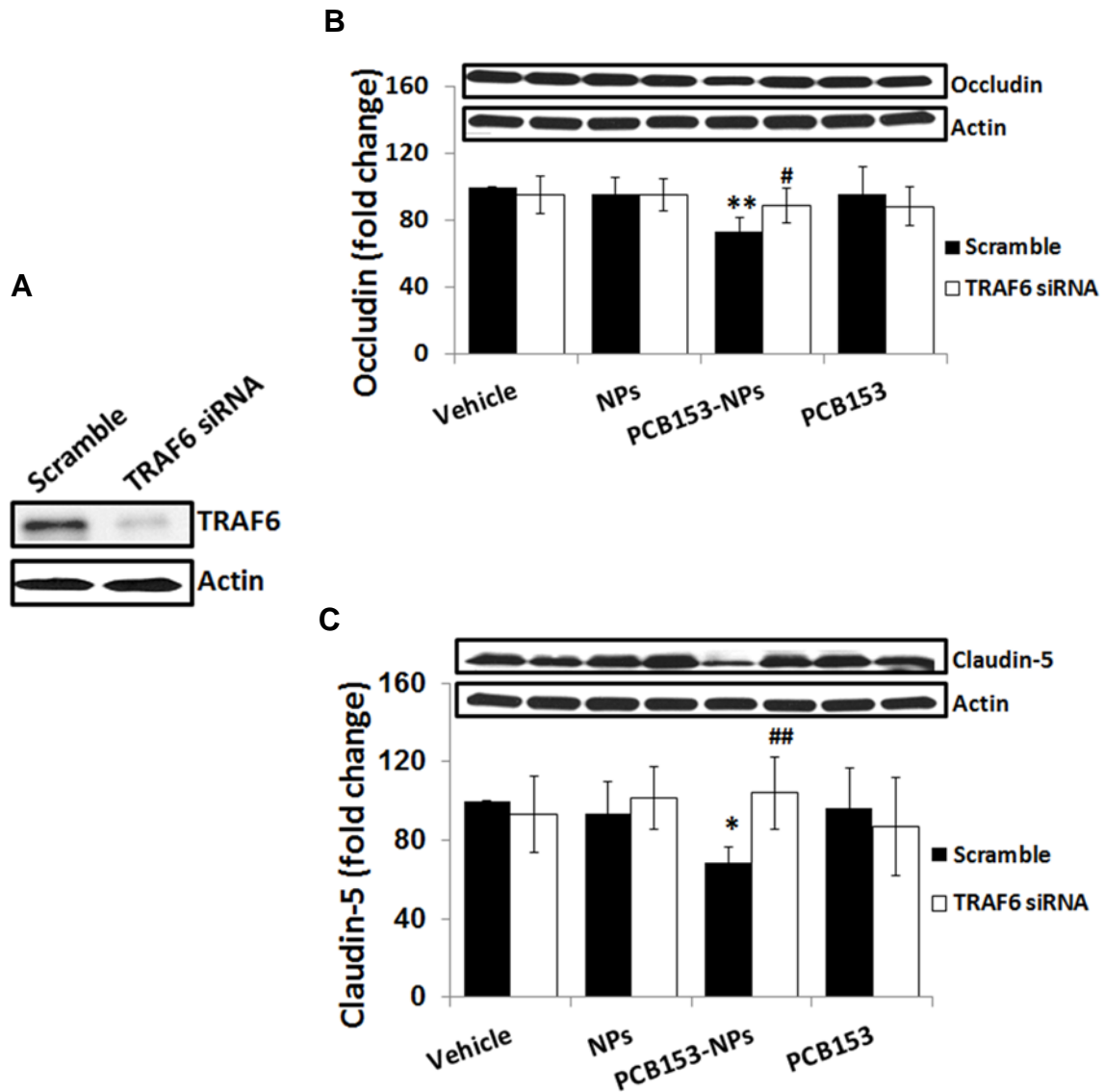


Figure 4.5. TRAF6 mediates PCB153-NP-induced a decrease in TJ protein expression. (A) Immunoblotting illustrating the efficiency of TRAF6 silencing. (B and C). Human brain endothelial cells were transfected with TRAF6 siRNA, followed by treatment with PCB153 and/or NPs as in Figure 4.2B for 24 h. Expression of occludin (B) and claudin-5 (C) was assessed by immunoblotting. The blot illustrates the representative data of four independent experiments and the bar graph shows quantified results. Results are means \pm SEM. Significantly different as compared to vehicle at $*p<0.05$ or $**p<0.01$. Results in cultures with silenced TRAF6 are statistically different from those in the corresponding cultures transfected with control (scrambled) siRNA at $^{\#}p<0.05$ or $^{\#\#}p<0.01$.

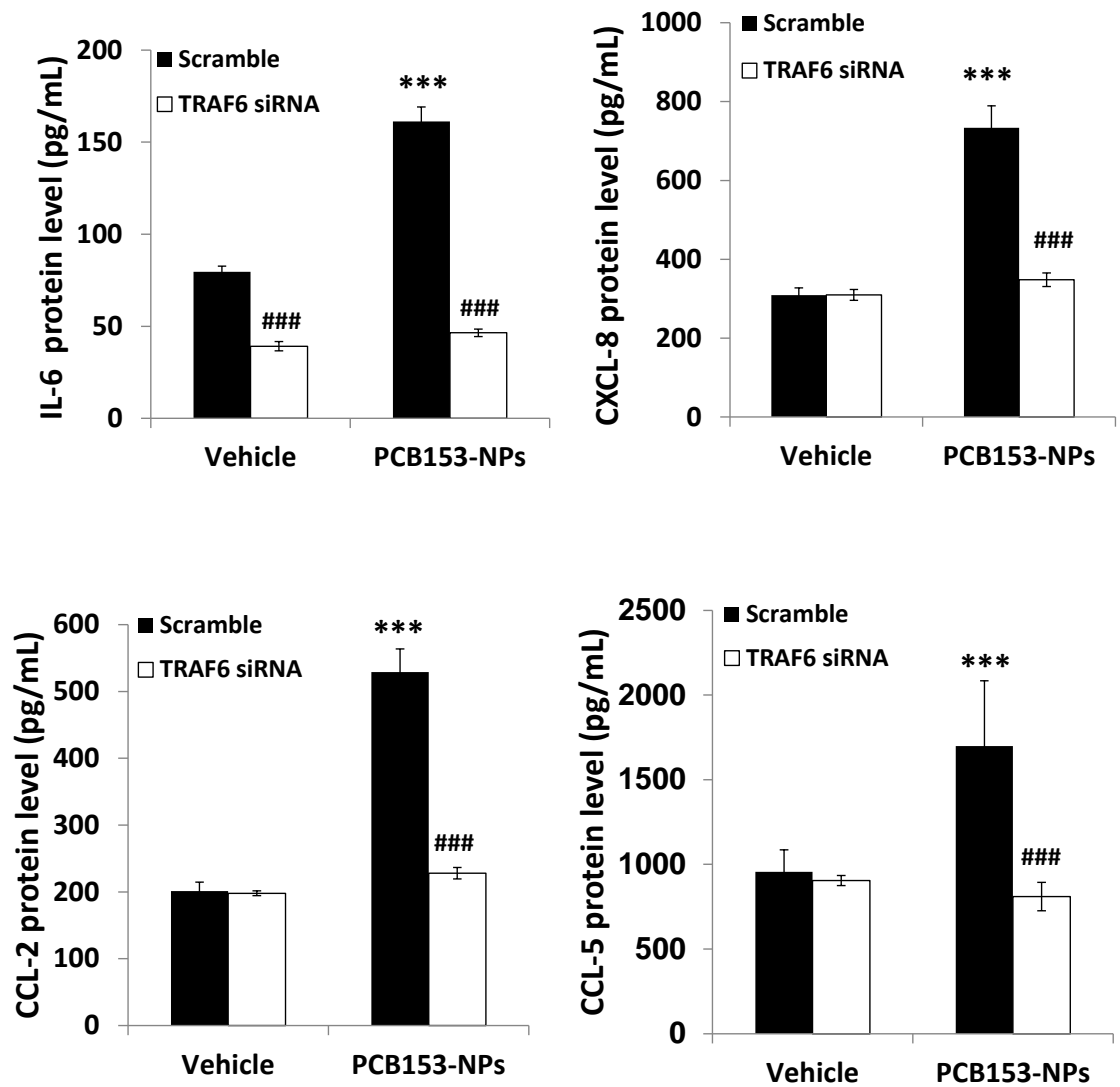


Figure 4.6. TRAF6 mediates PCB153-NP-induced production of inflammatory mediators. Human brain endothelial cells were treated as in Figures 4.5B and C, followed by the assessment of IL-6, CXCL-8, CCL-2 and CCL-5 in culture medium by ELISA. Results are means \pm SD, n=4. Significantly different as compared to vehicle at ***p<0.001. Results in cultures with silenced TRAF6 are statistically different from those in the corresponding cultures transfected with control (scrambled) siRNA at ###p<0.001.

5. Discussion

While the cellular effects of dioxin-like PCBs are linked to activation of the aryl hydrocarbon receptor (AhR), signal transduction mechanisms induced by *ortho*-PCBs are complex and include more diverse number of receptors and signaling pathways. Non-coplanar PCBs, such as PCB153 used in the present study, possess at least two *ortho* chlorines on the biphenyl ring, which generate steric forces that rotate the ring structure away from a single plane. Such a structure precludes interactions with the AhR; however, *ortho*-PCB congeners can act as ligands for the constitutive androstane receptor (CAR) and/or the pregnane-X receptor (PXR), and activate genes targeted by these receptors [307]. In addition to the nuclear receptors, ryanodine receptors (RyRs) have also been identified as candidates to mediate *ortho*-PCB-induced perturbations in cellular Ca^{2+} signaling, which plays a pivotal role in metabolism, proliferation, gene transcription, and protein translation in almost all cell types [308]. For example, PCB95 and PCB153 at concentrations lower than 1 μM were shown to significantly enhance activity of RyR1 and RyR2 [309]. Furthermore, *ortho*-PCBs can activate several signaling cascades including Janus kinase (JAK), epidermal growth factor receptor (EGFR), Src kinase, and mitogen-activated protein kinase (MAPK) [142, 310, 311]. We demonstrated that PCB153 upregulates expression of ICAM-1 and VCAM-1 through the Src/JAK/EGFR redox signaling, which is triggered by the NADPH oxidase-mediated increase of superoxide generation [143].

In the current study, we present evidence that TLR4 is yet another cellular receptor that is involved in *ortho*-PCB-mediated vascular toxicity [312]. While it is generally accepted that TLRs are sensors of a variety of biological molecules, like polysaccharides, proteins and nucleic acids [313], our observations that a non-biological material, such as PCB153-NPs, can act via the TLR4 signaling pathway are novel. By inhibition of TLR4 activity via pharmacological inhibitors and by using TLR4 deficient mice, we demonstrated that proinflammatory effects of PCB153-NPs are sensed via TLR4 in both brain microvessels and brain endothelial cells. These proinflammatory mediators are actively involved in the development of cerebrovascular and neurovascular alterations. ICAM-1 is an adhesion molecule which stimulates firm adhesion of leukocytes to the vascular endothelium and plays a critical role in the pathology of

numerous proinflammatory vascular diseases, including atherosclerosis [314]. CXCL-8 is one of the CXC chemokine members that has potent chemotactic activity for neutrophils [315]. It has also been shown that CXCL-8 can induce generation of superoxide and hydrogen peroxide [316] as well as increase expression of adhesion molecules [317, 318]. CC chemokines, such as CCL-2 and CCL-5, are implicated in the activation of monocytes, macrophages and lymphocytes [319]. Additionally, CCL-2 stimulates monocytes to produce tissue factor and proinflammatory cytokines, including IL-6 [36]. An elevated IL-6 level is associated with an increased infarct volume and severity of stroke outcome [320, 321].

Activation of TLR4 results in interaction of its intracellular TIR domain with MyD88, whose amino-terminal death domain (DD) associates with the serine kinase IL-1 receptor-associated kinase (IRAK). These events subsequently recruit TRAF6 [299], followed by nuclear translocation of proinflammatory transcription factors NF- κ B and AP-1. In agreement with this general pathway, we observed that treatment of brain endothelial cells with PCB153-NPs resulted in binding of TRAF6 to TLR4. While these interactions were transient, their importance was evident as silencing of TRAF6 significantly attenuated PCB153-NP-induced overproduction of inflammatory mediators.

Although the involvement of TLR4 in modulating BBB disruption has been reported [322], the precise mechanisms involved are not fully understood. Therefore, our observation that TLR4 signaling modulates PCB153-NP-induced disruption of TJ protein expression is another novel finding in the current study. We propose that TLR4 mediated an increase in inflammatory mediators, which may be responsible, at least in part, for these effects. Indeed, CCL-2 has been reported to induce occludin phosphorylation on both serine/threonine residues, resulting in increased BBB permeability. Furthermore, CCL-2 targets ZO-1 and claudin-5 phosphorylation through a signaling pathway involving Rho and protein kinase C (PKC) [53, 323]. It was also reported that TLR4/TRAF6 signaling is involved in nanomaterial-induced autophagy formation [324]. While autophagy is a highly conserved pathway of intracellular protein degradation [325, 326], our laboratory provided evidence that stimulation of autophagy in brain endothelial cells is associated with decreased expression of the TJ proteins [277].

Dysregulation of TLR4 signaling appears to be involved in several disorders, including cerebral ischemia and stroke [180, 202]. Consistent with these reports, we observed that the infarct volume in TLR4-deficient mice treated with PCB153-NPs was significantly decreased as compared to mice with normal expression TLR4. While a variety of factors can contribute to the development of stroke, the pathology of ischemia/reperfusion has a very strong inflammatory component [327]. Therefore, inflammatory responses induced by PCB153-NPs in cerebral vessels are likely to be responsible for the development of enhanced brain infarct. BBB breakdown, due to disruption of TJs and infiltration with inflammatory cells, may be another contributing factor to the progress of the brain injury following ischemia/reperfusion and exposure to PCB153-NPs.

In summary, our study demonstrates that exposure to PCB153 bound onto silica nanoparticles triggers TLR4/TRAFF6-regulated inflammatory responses and alterations of TJ protein expression, which then contribute to enhanced brain injury following ischemia/reperfusion (Figure 4.7). These results indicate an important role for TLR4 signaling in PCB-mediated cerebrovascular toxicity, suggesting that this signaling pathway may be a potential target for therapeutic intervention in cerebrovascular disorders.

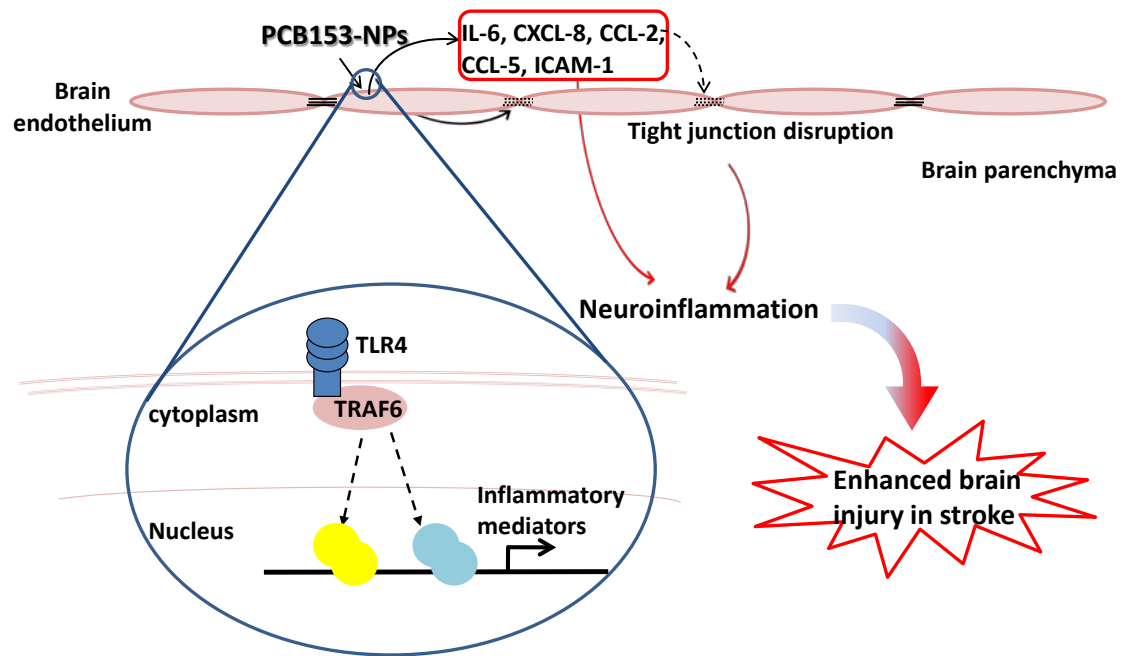


Figure 4.1. Schematic diagram of PCB153-NP-induced cerebrovascular toxicity via stimulation of the TLR4. Binding of PCB153 to silica nanoparticles activates the TLR4 and triggers its interaction with downstream molecule TRAF6. These events subsequently initiate overexpression of proinflammatory mediated and decreased expression of TJ proteins in brain endothelial cells. Disrupted BBB and enhanced neuroinflammatory responses contribute to enhanced brain injury in experimental stroke model.

Chapter five: Overall discussion

1. Summary

The work presented in this dissertation provides the evidence that disruption of the BBB contributes to the brain ischemic injury. This phenomenon was evaluated in models of telomerase deficiency animals and mice exposed to environmental pollutants, such as PCBs and NPs. The BBB, which is far from being just a simple physical barrier, is an intricate, heterogeneous and dynamic tissue. The normal BBB integrity controls cerebral homeostasis, while dysfunction of the BBB contributes to the formation and progression of CNS diseases.

Differences in age, gender, genetic background, and environment can influence the BBB in delicate ways. Although declines in BBB function may be multifactorial, aging and aging-related defects largely contribute to BBB impairment. Moreover, diseased conditions at the late stage of life may cause additional damage in the brain. Telomere shortening and telomerase deficiency are closely associated with accelerated endothelium aging. As presented in our work, deficiency of telomerase resulted in significantly pronounced BBB disruption and cerebral ischemic injury following the onset of stroke.

In addition to the endogenous factors, BBB integrity is affected by exogenous stimuli. Since the brain endothelium forms an interface between the blood and CNS, it may be easily targeted by a variety of environmental pollutants, including PCBs and NPs, which enter human body in various manners and circulate in the blood. PCBs and/or NPs-mediated brain endothelium dysfunction, including overproduction of inflammatory mediators, transmigration of monocytes, and BBB impairment are highly linked with increased vulnerability to stroke-related injury. In fact, our studies also demonstrate that exposure to PCB-bound particles exacerbate brain damage following ischemia/reperfusion.

2. Telomere and telomerase in age-associated vascular dysfunction and vascular diseases

Telomere biology in cancer has been well studied over the decades; however, the role of telomere in the field of vascular biology and aging-associated vascular diseases, including stroke, has been partially investigated in the recent years.

The presence of telomerase activity in the brain during embryonic development characterizes the role of telomerase in neuronal differentiation and survival [224]. Both telomerase and TERT levels are generally decreased during embryonic and early postnatal development. However, telomerase activity declines dramatically as the neuroblasts stop proliferation and start differentiation, whereas the level of TERT remains high in this critical period and then decreases in association with the natural cell death [83]. This unparalleled change between telomerase activity and TERT levels suggests that TERT may function to promote cell survival through a mechanism other than maintaining telomere length [225]. In addition, recent studies have demonstrated that TERT exerts many other functions, including metabolism, secretion of growth factors, energy balance, mitochondria function, and apoptosis [328]. Importantly, the induction of telomerase in response to brain injuries have been shown in each of the major cell types in the brain, including neurons, astrocytes, microglia and neural stem cells [86, 210, 329], suggesting the intriguing possibility that TERT has protective effects of preventing cell death in response to hypoxia or brain injury.

Telomerase-deficient mice are crucial models for evaluating the disease states associated with both normal aging and premature aging syndromes. The first generation of telomerase-deficient mice have been found to exhibit a normal phenotype, which is most probably due to the regular length of the telomere [330, 331]. However, telomere attrition with the successive generations results in infertility at the sixth generation because of the impairment of the reproductive system. Additionally, late-generation mice show aging-related phenotypes. For example, these animals have been reported to exhibit a compromised capacity in response to stress such as wound healing and hematopoietic ablation [332]. It has also been shown in the late generations of telomerase-deficient mice that the ability of neovascularization is reduced as compared to control animals [333]. In

the current study, we used TERT^{-/-} mice of the second, third, and fourth generations. There were no apparent changes in body weight, lifespan or behavior (e.g. food uptake) between TERT^{-/-} mice and wild-type animals under normal physiological conditions. However, upon the onset of brain ischemia, TERT^{-/-} mice appeared to be more susceptible to the stress as exhibited by severe neurological deficits at 24 h, impaired recovery, and higher mortality as compared to wild-type mice. In contrast, TERT transgenic mice showed significant resistance to ischemic brain injury [86]. Additionally, TERT transgenic mice exhibited a reduced myocardial infarct area following coronary artery ligation, and exogenous expression of TERT promoted cardiac myocyte survival [212]. These findings indicate that TERT ^{-/-} mice are more susceptible to the development of stroke and TERT expression can markedly influence the survival of both neurons and non-neuronal cells.

While telomeres shorten during cell divisions in cultured human endothelial cells and vascular smooth muscle cells (VSMCs), introduction of TERT can extend the lifespan of these cells, suggesting an important role of telomere shortening in the senescence of vascular cells and aging-related vascular disease [334]. It has been reported that in the endothelium of the abdominal aorta and iliac arteries, the length of telomeres and the age show a significant inverse correlation [335, 336]. Additionally, telomere length in white blood cells from patients with severe coronary artery disease is significantly shorter than that of controls. This may reflect not only increased leukocyte turnover resulting from chronic inflammation associated with atherosclerosis, but also accelerated cell turnover in other tissues including vascular cells [337]. Moreover, it has been shown that dysfunction of telomerase markedly increases the expression of ICAM-1 and reduces the activity of eNOS [236]. In agreement with these findings, our results in the current study illustrate that TERT deficiency potentiate the pMCAO-induced increase of ICAM-1, TNF- α and IL-1 β expression in the mouse brain. It was also demonstrated that TNF- α -mediated increase in monocyte adhesion in senescent human endothelial cells was attenuated in TERT transfected cells [236], further indicating a relation between telomerase activity and endothelial dysfunction.

Another novel observation in the present study indicates that TERT deficiency potentiates the oxidative stress in the mouse brain capillaries following the induction of pMCAO. Oxidative stress has been implicated in human aging as well as cellular senescence. In addition, increased oxidative stress is known to stimulate redox-regulated signaling pathways that deregulate TJ protein expression and affect the BBB integrity [237]. The process of endothelial cell aging, accompanied by the diminishing of TERT activity and proceeding to the replicative senescence, is associated with the increase of ROS generation. It has been suggested that increased oxidative stress results in nuclear export of TERT into the cytosol in endothelial cells depending on the Src family kinase activation, whereas treatment with antioxidants and statins can block the reduction of nuclear TERT activity [246]. Furthermore, repression of oxidative stress maintains the telomere length and elongates cell lifespan, which is partially through the activation of telomerase [242]. It has also been proven that NO can activate telomerase and prevent the endothelial cells from senescence. One of the possible explanations is that decreased oxidative stress, which happens when NO reacts with cellular radicals, triggers telomerase activation.

Aging is one of the major risk factors for stroke and other vascular diseases. Taken together, it is suggested that telomere biology is important for the function of endothelial cells, and shortening of telomere or dysfunction of telomerase may contribute to the pathophysiology of vascular aging. However, the mechanisms by which telomerase dysfunction causes vascular dysfunction and aging-related vascular diseases have not been fully understood. Future challenges will be to develop and improve approaches to test the cellular senescence and telomere/telomerase dysfunction in vascular system aging *in vivo*. It is hoped that telomere/telomerase-based prevention will be utilized to as a promising target to combat aging-related vascular diseases, including stroke.

3. Potentiated cerebrovascular toxicity of particle-bound PCBs

Environmental pollution, in particular PMs and POPs, has been recognized as a risk factor for vascular disease and stroke. Specifically, elevated environmental levels of

PCBs are associated with increased levels in serum in the exposed population and higher prevalence to disease development, such as diabetes and hypertension [132, 256], which are associated with stroke. Although only limited data on the association between ambient air pollution and cerebrovascular disease has been reported, exposure to different components of air pollution, such as PMs, ozone, carbon monoxide, and nitrogen dioxide, is highly linked with an increased incidence of cerebrovascular ischemia [148, 154, 170].

Results from animal experiments indicated that ultrafine PMs can translocate into the systemic circulation and the brain through inhalation or nasal instillation [52, 267]. Thus, particulate components from the ambient air pollution may act as effective carriers for various environmental pollutants entering the brain. PCBs are semivolatile organic compounds. Due to their volatility and persistence, they are subjected to long-range atmospheric transport and capable of binding onto different sizes of PMs in the environment and taken up by humans through ingestion and/or inhalation. However, research concerning the adverse health effect of particle-bound PCBs is currently limited. Our studies provide evidence indicating that PCB153-NPs may have higher cerebrovascular toxicity as compared to the same dosage of PCB153 exposure, which is proved by the significantly disrupted BBB integrity, markedly increased inflammatory responses, and potentiated brain injury following ischemia/reperfusion. The toxicological properties of nanoparticles depend on various parameters, such as size, dose, dimension, surface chemistry, aggregation, etc. It is not a simple additive process to estimate the risk of two or more pollutants. The surface of the nanoparticles plays an important role in toxicity since it makes direct contact with cells and tissues. Surface coating can make noxious nanoparticles harmless or render relatively safe nanoparticles toxic. For example, the presence of oxygen radicals, ozone and transition metals on the surface of nanoparticles creates ROS and induces inflammation [338-340]. It has been reported that exposure to diesel exhaust particles interacting with ozone induces elevated inflammatory responses in the animal lung as compared to diesel particles alone [338]. Another *in vivo* experiment showed a more prominent formation of blood clots in animals exposed to surface aminated polystyrene nanoparticles [341]. In contrast, the spherical gold nanoparticles with different surface coating exhibit non-toxic effects to human cells [342, 343]. Additionally, nickel ferrite nanoparticles have been demonstrated to show different

cytotoxicity with and without surface coating of oleic acid [344]. Thus, the advanced vascular toxicity of PCB153-NPs may result from the synergic effects of both compounds. Secondly, the properties of nanoparticles as effective carriers across the BBB are well described [285]. Hence, binding to silica nanoparticles may facilitate PCBs crossing the BBB and effectively reaching the brain parenchyma. In addition, our long-term study shows a higher PCB153 body burden in animals chronically exposed to PCB153-NPs than in PCB153 alone. One of the possibilities is that PCB153-NPs have a lower excretion rate due to the longer retention of nanoparticles [286]. Another explanation is the effects of PCB153 and/or nanoparticles on intestinal epithelium, which may contribute to increased PCB153 absorption into the blood stream. In fact, ingestion of PCBs is the primary exposure route in humans. Our laboratory has already demonstrated that oral exposure to PCBs can disrupt TJ proteins in the small intestine and increase intestinal permeability [278].

The main toxic effects induced by coplanar PCBs are mediated via binding to AhR and expression of downstream genes [345]. However, the signal transduction mechanisms induced by ortho-PCBs are complex and include more diverse numbers of receptors and signaling pathways. It has been reported that ortho-PCB congeners can act as ligands for the constitutive androstane receptor (CAR), the pregnane-X receptor (PXR) [307], and ryanodine receptors (RyRs) [308]. According to our present findings, TLR4 is yet another cellular receptor that is involved in ortho-PCB-mediated vascular toxicity. Recently, TLR4 has been increasingly recognized as a target receptor in response to the stimuli of various environmental pollutants. Results from a quantitative trait locus analysis indicate that, in very few practicable candidate genes on chromosome 4, TLR4 is involved in lung hyperpermeability in mice in response to ozone exposure [204]. This finding raises the probability that a molecule considered as a key component in innate immunity by sensing and regulating responses to endotoxin or LPS can also mediate the responses to environmental pollutants. The involvement of TLR4 in response to ozone exposure has been proven in studies using TLR4-deficient mice, whose hyper-reactivity following sub-chronic ozone exposure was markedly decreased as compared to the wild-type control animals [346]. TLR4 signaling has not only been suggested to have a role in sensing the environmental pollutants, it has also been indicated to modulate the outcomes

of ischemic injury [180, 202]. In agreement with these findings, our results show that deficiency of TLR4 or inhibition of TLR4 signaling pathway significantly abrogated PCB153-NP-induced enhancement of ischemic brain injury, increase of inflammatory response, and disruption of TJ protein expression *in vivo* and *in vitro*. Our results indicate that TLR4 may be a potential target for investigating neurotoxicity associated with environmental pollutants, such as PCBs and/or NPs, and the progression of cerebrovascular disease.

Although the production has been halted for approximately 50 years, PCBs are still ubiquitous in the environment. Recent research mainly focuses on the toxic effects and mechanisms of individual PCB congeners in different biological systems. However, since the transport modes of PCBs in the environment are multifarious, studying the effects of PCBs assembled onto environmental particles may provide new perspectives for assessing the toxicological profiles of PCB exposure. In addition to PCB153 that is investigated in the current work, other PCB congeners, such as dioxin-like PCBs, have been detected on the environmental particles, thus investigation of the health effects of different PCB congeners bound to NPs is also necessary. Furthermore, other than TLR4, various molecules have been suggested as candidates to sense and respond to ambient air pollutants. For example, members of the glutathione S-transferase superfamily of phase II xenobiotic metabolizing enzymes, including GSTP1 and GSTM1, are ideal candidate genes to examine the variation in humans in responsiveness to diesel exhaust particles (DEPs) [347]. In the future, identification of susceptible targets for various environmental pollutants may be emergent for health assessment and therapeutic purposes.

4. Future directions—targeting the BBB

The work presented here offers intriguing insights of modulating BBB function by different factors. Due to the involvement of BBB disruption as an early event in neurological conditions, targeting the BBB may provide insight for the prevention and treatment of CNS disease. In this dissertation, we mainly focus on the functional alterations of brain endothelium in response to the stress of environmental pollutants (e.g.

PCBs and NPs) and a diseased condition—cerebral ischemia. The brain endothelium contributes to the physical and chemical barrier properties. In fact, the BBB is dynamic, with a big range of permeability, which is adjusted by intracellular and intercellular events among not only endothelial cells, but also astrocytes, pericytes, and neurons. Because of the complex barrier properties and the anatomical relations of the cells engaged in the BBB, it is not surprising to find that the modulation of cerebral vasculature involves the synergistic inductive functions of more than one cell type. For example, it has been reported in an *in vitro* study that astrocytes are needed for the proper association of endothelial cells and pericytes in tube-like structures [348]. Under conditions such as physiological levels of neuronal activity or pathological stimuli [349, 350], cross talk between neurons and glial cells, especially astrocytes [351], can influence cerebral blood flow. In addition to the NVU, a recent study indicates that interactions between leucocytes and brain endothelium play an important role in the initiation of seizure activity.[352]. This key observation not only signifies the importance of the BBB, but also highlights the need of considering blood cells and inflammatory signals as key players in initiating CNS pathology. Since blood cells and inflammatory signals are important mediators of CNS pathophysiology, it has been proposed that they should be considered as part of the ‘extended NVU’ [353]. Our results indicate that inflammatory responses may be one of the major contributors of PCB-mediated brain endothelium dysfunction. We demonstrate that both acute and chronic exposure to PCB153-NPs significantly upregulated the interaction of monocytes and leukocytes with the brain endothelium, a key event that contributes to the CNS pathology. Thus, other than brain endothelial cells, the synergistic effects within NVU, as well as the contribution of blood cells, should be considered as part of the future investigations of BBB study.

One of the challenges in BBB research is to find better ways of imaging and monitoring functions of the living brain. In animal stroke models, opening of the BBB can be detected as early as minutes following occlusion of the middle cerebral artery [354]. Therefore, BBB disruption may stand as the early and decisive step in the acute cerebral ischemic stroke [355, 356]. So far, extravasation of certain dyes, such as Evan’s blue, is commonly used in animal studies as an index of BBB permeability that is also correlated with the brain infarct size. In future studies, the application of non-invasive

methods such as dynamic magnetic resonance, which images cerebral perfusion, and functional magnetic resonance imaging (fMRI), which detects signal intensity via changes in local blood flow and oxygenation, should be considered in the animal models to study the CNS etiology. With nanoparticle-based brain mapping technology, these methods can be further advanced to help us understand the neuro-glio-vascular coupling and BBB pathophysiology [357]. Moreover, changes in brain endothelial transporters also indicate the alteration of the BBB permeability. Recently, the *in vivo* assessment of specific BBB transport systems has been achieved by using positron emission tomography (PET). Studies using a radiolabeled P-glycoprotein (P-gp) ligand have evaluated the endothelial P-gp function and its role in the uptake and binding of drugs in the intact CNS [358]. At the microscopic level, the bioimaging technologies allow the *in vivo* observation of tracking of exogenous cells, including tumor cells, immune cells and progenitor cells. These technologies have greatly enhanced our knowledge of the behavior of different circulating cells across the BBB in different models of CNS disease, such as ischemic brain injury, autoimmune demyelination, and brain metastasis [359]. By using the fluorescence microscopy in the present study, we successfully visualized the leukocyte-endothelium-interaction in the living mouse brain, which further validated the inflammatory responses and vascular toxicity provoked by exposing to PCB153-NPs. In the future, surface modified nano-materials such as fluorescence-conjugated nanoparticles, could be applied *in vitro* and *in vivo* to determine the cellular and tissue uptake of PCB-bound nanoparticles. Additionally, for the purpose of early diagnosis or monitoring of disease, molecular changes in the NVU could be developed as imageable biomarkers using targeted molecular imaging agents [360]. However, since there is no single method able to capture different orders of magnitude in temporal and spatial resolutions while showing cellular and vascular events, it is necessary to develop better ways to combine and integrate data obtained from multi-modal imaging techniques.

BBB disorders are involved in early phase of numerous CNS pathologies. Thus, targeting the BBB for the early treatment may help to alleviate the severity of neuro-pathological symptoms and facilitate recovery. Since the complexity of BBB regulation is not fully understood, further investigations, such as detailed mechanisms involved in the communication among NVU in response to stress, as well as improved techniques for

bioimaging, could advance our knowledge of the BBB and help in the design of therapies for CNS diseases.

5. Conclusions

In conclusion, findings in this dissertation demonstrated that BBB properties can be affected by both endogenous and exogenous factors. Deficiency of telomerase activity, a key event associated with accelerated aging, aggregates cerebral ischemic stroke-induced brain damage through disruption of BBB integrity and potentiation of neuroinflammatory responses. In addition, the cerebrovascular toxic effects of PCBs, one of the most persistent organic pollutants, can be enhanced by binding to nanoparticles, and the vascular toxicity of particle-bound PCBs is mediated through TLR4 signaling. Furthermore, our results indicate that impairment of BBB functions facilitates stroke outcomes. Findings resulting from the present dissertation may be applicable not only to stroke, but also to other neuropathological disorders where telomere biology, environmental pollutants, and/or BBB integrity play crucial roles.

Appendix Methods

1. Surgical Procedures

1.1. Procedure for middle cerebral artery occlusion (MCAO)

- Autoclave all the surgical instruments and materials prior to surgery.
- Mice were anesthetized with 1.5%~2% isoflurane in oxygen. After fixing the animal to the surgical platform using an adhesive tape, a midline neck incision was made under the operating microscope, and the soft tissues over the trachea were removed.
- The left common carotid artery (CCA) was carefully isolated from the vagus nerve and ligated using a 6-0 silk thread.
- Then, a permanent knot was placed at the distal part of the external carotid artery (ECA) to prevent the backflow of blood, and a vessel clip was temporarily placed on the internal carotid artery (ICA) close to the CCA junction.
- The tied section of CCA was incised using the microscissors to insert the silicon coated filament. Once the tip of the filament reached the CCA junction, the vessel clip placed on the ICA was removed to allow filament insertion.
- The filament was advanced carefully up to 10 ± 1 mm into the MCA from the CCA junction.
- Once filament insertion into the MCA was confirmed, the mouse body temperature was maintained at 37 °C using a heating blanket during occlusion.
- After a 40 min occlusion period, the filament was then withdrawn carefully to restore the blood flow.
- After the removal of the filament, the knot was tightened at the CCA. Then, the midline neck incision was sewed using surgical suture.
- For sham surgery, all the arteries were exposed for the surgical period but no filament is inserted into the MCA.
- At the end point of the study (24h following reperfusion), the animals were sacrificed and histological analysis was carried out by using 2% 2,3,5-triphenyltetrazolium chloride (TTC) staining to confirm the brain infarct volume.

1.2. Procedure for vessel tubule insertion

- All the surgical instruments and materials were autoclaved prior to surgery.
- Mice were anesthetized with 1.5%~2% isoflurane in oxygen. Then, a midline neck incision was made, followed by isolation of the left CCA, ECA, and ICA under the operating microscope.
- The ECA was then ligated with 6-0 silk thread distal from the ICA.
- Two vessel clips were placed on CCA and ICA respectively to restrict the blood flow.
- A small incision was then made on the tied section of ECA, and then the tip of the tubing was inserted through the incision and advanced toward the CCA until it reached the bifurcation point.
- The tubing was secured with an additional knot around the ECA. Then, the vessel clips on the CCA and the ICA were removed before performing the injection.
- Treatment reagents were slowly instilled into the animal body through the inserted vessel tubule. After the injection, the tip of the tubule is gently removed from the ECA and a knot is tightened at the incision part of the ECA.
- Then, the midline neck incision is sewed using surgical suture.

2. Mouse cerebral microvessel isolation and immunofluorescence staining

- Isolation buffer A

NaCl -- 103 mmol/L

KCl -- 4.7 mmol/L

CaCl₂ -- 2.5 mmol/L

KH₂PO₄ -- 1.2 mmol/L

MgSO₄ -- 1.2 mmol/L

HEPES -- 15 mmol/L

Adjust pH to 7.4

- Isolation buffer B

NaCl -- 103 mmol/L

KCl -- 4.7 mmol/L

CaCl₂ -- 2.5 mmol/L

KH₂PO₄ -- 1.2 mmol/L

MgSO₄ -- 1.2 mmol/L

HEPES -- 15 mmol/L

NaHCO₃ -- 25 mmol/L

Glucose 10 mmol/L

Sodium pyruvate -- 1 mmol/L

Dextran (MW 64 K) -- 10 g/L

Adjust pH to 7.4

- Following decapitation, the mouse brain was removed from the skull and immediately immersed in ice-cold isolation buffer A with protease inhibitor (Roche Diagnostics, Indianapolis, IN).
- Brain stem, cerebellum, choroid plexus, and meninges were removed, and brains were homogenized in 5 mL of ice-cold isolation buffer B with protease inhibitor.
- Then, 7 mL of 26% dextran was added to the homogenate and fully vortexed, and the samples were centrifuged at 5,800 x g at 4 °C for 20 min.
- The supernatant was discarded, and the collected pellet was resuspended in 7 mL isolation buffer B and filtered through a 70 µm cell strainer (BD Biosciences, San Jose, CA).
- Filtered homogenates were re-pelleted by centrifugation at 1500 x g 4 °C for 10 min and smeared on positively charged microscope slides (Fisher Scientific, Houston, TX).
- Smeared cerebral microvessels on the glass slides were allowed to dry at 100 °C for 10 min, and then fixed with 3% formaldehyde in phosphate-buffered saline (PBS) for 10 min at room temperature.

- Slides were then washed with PBS five times, followed by permeabilization with 0.1% Triton X-100 for 30 min.
- The slides were then re-washed in PBS five times, and blocked in 1% bovine serum albumin (BSA) in PBS for 30 min at room temperature.
- Slides were incubated in primary antibodies (e.g. anti-occludin and anti-claudin-5 antibodies) diluted in antibody diluent with background reducing components (Dako, Carpinteria, CA) overnight at 4°C.
- Slides were then rinsed in PBS five times, followed by incubation in FITC-conjugated goat anti-mouse IgG or Texas red-conjugated goat anti-rabbit IgG (1:1,000 dilution) for 1 hr at room temperature.
- All slides were mounted with ProLong Gold Antifade reagent containing 4',6-diamidino-2-phenylindole (DAPI) to visualize the nuclei, and coverslipped with cover glass.

3. BBB permeability assay.

- Animals were injected i.p. with 200 μ L 10% sodium fluorescein in PBS.
- Fifteen minutes later, blood was collected via cardiac puncture. Animals were then transcardially perfused with 0.9% saline and brains were harvested.
- Each brain sample was then weighed, homogenized in 1/10 dilution in PBS (w/v) and centrifuged at 14,000 xg for 5 min.
- Then, 500 μ l supernatant was transferred into 500 μ l of 15% trichloroacetic acid and centrifuged at 1,000 xg for 10 min.
- After centrifugation, 125 μ l of 5 N NaOH was added to 500 μ l of the supernatant and mixed well. Then, 200 μ l of the mixture was loaded in 96-well plate for measurement.
- To remove the hemoglobin contamination from the plasma, 15% TCA was added to plasma samples as the ratio of 1:10. The mixture was vortexed and centrifuged at 1,000 x g for 10 min.
- Then, add 5 N NaOH to the resulting supernatant as the ratio of 1:4. Dilute the fluorescein contained supernatant with PBS (1:100) if the fluorescence value is too high. Then 200 μ l of the mixture was loaded in 96-well plate for measurement.

- A standard curve for quantification of sodium fluorescein in the samples was generated by simultaneously analysing samples of known sodium fluorescein concentration in PBS.
- Fluorescence was determined using excitation at 485 nm and emission at 530 nm. The permeability results are presented as a ratio of brain to plasma fluorescence intensity. The index of BBB permeability was calculated as the ratio brain:plasma fluorescence

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Publications

1. **Zhang B**, Choi JJ, Eum SY, Toborek M. TLR4 signaling is involved in brain vascular toxicity of PCB153 bound to nanoparticles. (submitted to PLOSONE)
2. **Zhang B**, Chen L, Hennig B, Toborek M. Cerebrovascular toxicity of PCB153 is enhanced by binding to silica nanoparticles J Neuroimmune Pharmacol, 2012 Dec;7(4):991-1001. doi: 10.1007/s11481-012-9403-y. Epub 2012 Oct 19
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Abstracts and Presentations

1. **Zhang B**, Choi JJ, Eum SY, Toborek M. TLR4-mediated signaling is involved in PCB153-NP-induced cerebrovascular toxicity in the experimental stroke model. NIEHS Superfund Research Program 25th Annual Meeting, October 21-24, 2012, Raleigh, NC.
2. **Zhang B**, Chen L, Hennig B, Toborek M. Cerebrovascular toxicity of PCBs bound to nanoparticles in the experimental stroke model. Society of Toxicology, 51th Annual Meeting, March 11–15, 2012, San Francisco, CA.
3. **Zhang B**, Chen L, Hennig B, Toborek M. Cerebrovascular toxicity of PCBs bound to nanoparticles in the experimental stroke model. Superfund Research Program (SRP) Trainee Webinar, February 21, 2012. Oral Presentation
4. **Zhang B**, Chen L, Hennig B, Toborek M. Cerebrovascular toxicity of PCBs bound to nanoparticles in the experimental stroke model. NIEHS Superfund Research Program Annual Meeting, Lexington, Kentucky, October 24-26, 2011.

5. **Zhang B**, Chen L, Hennig B, Toborek M. Toll-like receptor 4 is involved in the cerebrovascular toxicity of PCB153 bound to nanoparticles. Society of Toxicology 50th Anniversary Annual Meeting, Washington, D.C., March 6 – 10, 2011
6. **Zhang B**, Chen L, Hennig B, Toborek M. Toll-like receptor 4 is involved in the cerebrovascular toxicity of PCB153 bound to nanoparticles. NIEHS Superfund Research Program Annual Meeting, Portland, Oregon, November 10-12, 2010
7. **Zhang B**, Chen L, Choi YJ, Hennig B , Toborek M. Silica nanoparticles coated with PCB153 alter the blood-brain barrier integrity and induce neuroinflammatory responses. Society of Toxicology, 49th Annual Meeting, March 7-11, 2010
8. **Zhang B**, Chen L, Choi YJ, Hennig B, Toborek. Silica nanoparticles coated with PCB153 alter the blood-brain barrier integrity and induce neuroinflammatory responses. NIEHS Superfund Research Program: Emerging Issues, Emerging Progress, New York, November 2-5, 2009
9. **Zhang B**, Chen L, Swartz KR, Bruemmer D, Hennig B, Toborek M. Deficiency of telomerase activity affects the blood brain barrier disruption and neurological outcome in a model of middle cerebral artery occlusion. Experimental Biology 2009 meeting, April 18th-22th, New Orleans USA, FASEB J. 23: 1020.9, 2009